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ERRATA

In Volume 7, Part 4 (December 1959) on page 562, the order of the figures 10·6 and 6·5 in the last column of the table should be reversed.

La compétition entre les éléments osseux du zeugopode de Poulet

par ARMAND HAMPÉ[1]

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EN traitant les bourgeons de patte de *Xenopus* avec une substance toxique, Tschumi (1954) observe une réduction progressive du nombre de doigts à mesure que la dose de substance toxique augmente. C'est d'abord le premier doigt qui disparaît, puis le quatrième et ensuite le deuxième. Le troisième doigt subsiste en dernier lieu. Cette règle s'observe aussi chez les Oiseaux. L'irradiation aux rayons X du bourgeon de patte par M. Schué (1951) conduit à une perte successive des 1er, 4ème, 2ème et 3ème doigts; cet ordre est toujours respecté. Étant donné que tous les éléments osseux présumés sont touchés et partiellement détruits par l'irradiation, on peut admettre que tous les éléments cellulaires disponibles, quelle que soit leur origine, sont mobilisés par priorité vers la formation du troisième doigt. S'il y en a davantage, ce sont les 2ème, 4ème et 1er doigts qui seront constitués.

Un phénomène analogue paraît se produire dans le zeugopode (article intermédiaire) de la patte de Poulet. Jusqu'aux environs du 5ème jour, le tibia et le péroné sont sensiblement de même longueur. Mais vers 6 jours d'incubation, le tibia distal pousse un diverticule latéral séparant le péroné du péronéal. Une fois que l'épiphyse distale du tibia s'est installée sur le côté postérieur de la patte, le péroné ralentit son accroissement alors que le tibia accélère son allongement. La croissance compétitive entre ces deux os est nettement à l'avantage du tibia qui, dans la suite du développement, devient un os complet, le péroné au contraire reste malingre et ne constitue qu'un reliquat filamenteux n'ayant aucun rôle sustentatoire.

Le péroné a même tendance à disparaître en cas de pénurie de matériel (14 cas sur 15), par exemple à la suite d'une excision de mésenchyme médian (Wolff & Hampé, 1954). Dans ce cas, tout le mésenchyme zeugopodial présumé sert à l'édification du seul tibia. La compétition est nettement plus âpre que dans le cas normal, puisque le péroné n'a même pas la possibilité d'apparaître. Wolff, Kieny & Schué (1958) ont abouti au même résultat par la méthode des irradiations aux rayons X. Dans tous les cas la disparition du péroné précède celle du tibia.

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On peut aussi réduire la compétition en agissant en sens opposé, c'est-à-dire en fournissant au bourgeon de patte un matériel supplémentaire. On met ainsi bout à bout la base d'un bourgeon âgé renfermant les ébauches du fémur, du tibia et du péroné et un bourgeon entier plus jeune renfermant les mêmes ébauches. La patte qui se développe à partir d'un tel complexe peut être normale, mais dans la majorité des cas (7 cas sur 8), le péroné dépasse sa longueur habituelle, atteint celle du tibia et se soude finalement au péronéal (Hampé, 1957; Wolff, 1958).

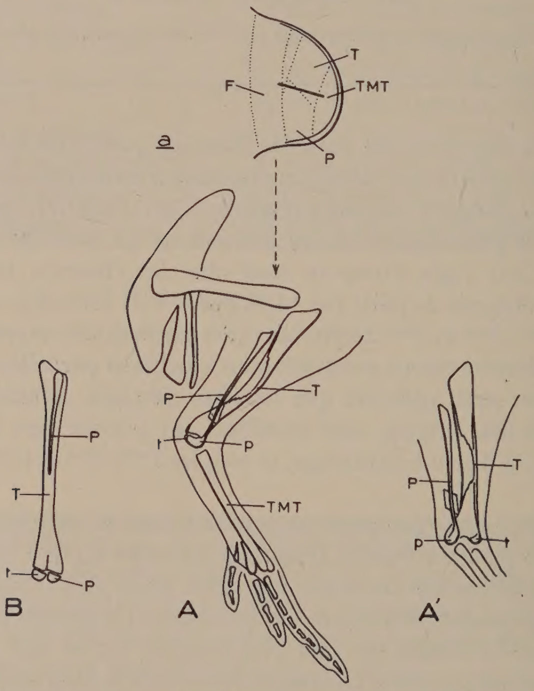

FIG. 1. Compétition entre le tibia et le péroné. *a*, la lamelle de mica est implantée entre les territoires des tibia (*T*) et péroné (*P*) présumés, mais selon une orientation qui se rapproche davantage de l'axe du bourgeon. *A*, le péroné (*P*) de la patte développée rejoint le péronéal (*p*), qui est commun avec le tibia (*T*). Le mica se retrouve entre les deux os du zeugopode. *A′*, le zeugopode vu de face. *B*, la patte gauche non opérée sert de témoin. *t*, tibial; *TMT*, tarsométatarsien.

L'excédent de matériel ne conduit cependant pas à un allongement du tibia. Celui-ci se développe comme d'habitude en se servant du matériel qu'il lui faut et, comme il en reste beaucoup, le péroné peut dépasser sa taille habituelle.

On peut essayer de supprimer la compétition entre les éléments du zeugopode, sans ajouter un matériel excédentaire. Deux possibilités sont offertes, la première consiste à implanter une lamelle de mica entre les ébauches présumées du tibia et du péroné. Dans la seconde, on découpe l'ébauche péronéenne à l'emporte-pièce et on la dévie d'une centaine de degrés de manière à faire diverger les directions de croissance des 2 os du zeugopode.

1. Dans deux cas la lamelle de mica est introduite à la limite entre le tibia et le péroné, mais selon une orientation qui se rapproche de l'axe du bourgeon. Le mica se retrouve dans la patte développée entre les tibia et péroné distaux. L'extrémité du péroné est très fine et se prolonge dans le péronéal. Le péroné plus long que d'habitude va de pair avec un tibia plus court que de coutume (Fig. 1).

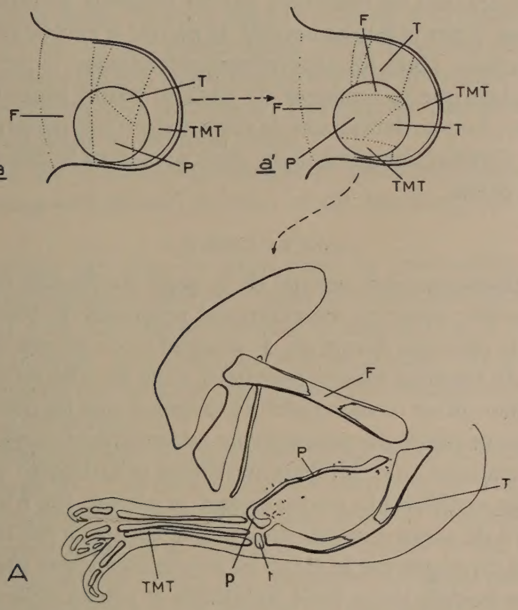

FIG. 2. Compétition entre le tibia et le péroné. *a*, le bourgeon au stade 23. On retourne l'ébauche du futur péroné. *a'*, la rotation de 90° intéresse le territoire du futur péroné (*P*) et des parties limitrophes du fémur (*F*), du tibia (*T*) et des tarsométatarsiens (*TMT*). *A*, le zeugopode et l'autopode sont désorientés; le péroné (*P*) est anormalement long et se soude au péronéal (*p*). Le tibia est plus court que d'habitude. *t*, tibial; *TMT*, tarsométatarsiens.

Dans certains cas, où le mica empiète sur le futur fémur, celui-ci est bifurqué et chaque épiphyse est articulée avec un os du zeugopode. Le péroné se raccorde cependant au péronéal.

Si le péroné est complet, c'est très vraisemblablement parce que le mica empêche le crochet latéral du tibia distal de s'insinuer dans la moitié postérieure du membre. En conséquence, le contact entre le péroné et le péronéal subsiste dans la patte développée.

2. La seconde technique, appliquée à 6 embryons, aboutit au même résultat.

Le tibia et le péroné, au lieu de se développer parallèlement, s'écartent, pendant le premier temps de leur formation, pour se retrouver par la suite; la séparation de ces deux pièces osseuses fait obstacle à l'implantation du crochet latéral du tibia entre le péroné et le péronéal; de ce fait, la rupture entre ces deux pièces n'est pas consommée et le péroné devient complet (Fig. 2).

Normalement, l'ossification endochondrale du péroné débute près de sa pointe distale; dans le cas du péroné complet, le centre d'ossification se situe au milieu de la diaphyse, c'est-à-dire à égale distance des 2 épiphyses. Ceci nous fait penser que le péroné est d'habitude incomplet, sa moitié distale étant accaparée par le tibia. Cette opinion est renforcée par les résultats quantitatifs des expériences précédentes. Chez le Poulet normal, le péroné n'excède pas les deux tiers de la longueur du tibia. Dans les expériences précédentes, le péroné dépasse sa taille habituelle alors que la longueur du tibia se réduit proportionnellement. Il est probable que, dans ce dernier cas, le péroné se sert du matériel qui se trouve de son côté pour s'édifier complètement, alors que, dans le cas normal, le tibia s'en attribue une partie.

CONCLUSIONS

Au cours du développement normal de la patte du Poulet, on assiste à une croissance compétitive entre les mésenchymes présumés du tibia et du péroné. C'est finalement le tibia qui donne un os complet et de grande taille, mais il se sert d'une partie du matériel mésenchymateux situé du côté du péroné. Celui-ci ne peut donc former qu'un os incomplet n'atteignant que les deux tiers du tibia.

Si on supprime une partie du mésenchyme intermédiaire, aussi bien tibial que péronéen, la compétition entre les deux territoires se fait sentir plus rigoureusement, car si le tibia se développe normalement, le péroné, lui, n'apparaît pas. Le matériel qui aurait dû servir à sa constitution est accaparé par le seul tibia.

Inversement, si on augmente le matériel mésenchymateux présent, le péroné se développe bien au-delà de sa taille habituelle et atteint celle du tibia qui ne subit aucune modification. Dans ce cas, l'excédent de matériel réduit la compétition.

On peut aussi supprimer la compétition en intercalant une lamelle de mica entre le territoire présumé du tibia et celui du péroné, mais selon une orientation correspondant sensiblement à l'axe du bourgeon. Le mica empêche le tibia de se servir d'un matériel autre que celui qui se trouve de son côté. De ce fait, il est un peu plus court que d'habitude; par contre, le péroné est nettement plus long et se soude au péronéal à son extrémité distale. L'allure de l'articulation du talon rappelle sensiblement celle de l'Archaeopteryx.

La compétition peut encore se supprimer en découpant et en retournant l'ébauche péronéenne de 90°; dans ces conditions, les deux os du segment moyen s'allongent dans deux directions opposées et aucune concurrence ne peut avoir lieu; les deux os du segment moyen de la patte sont de même longueur. Dans les deux cas, la destruction du cartilage débute au centre de la diaphyse. Ceci est inhabituel, car le péroné normal s'ossifie en premier lieu à l'extrémité distale de la diaphyse et ceci peut être considéré comme preuve que le péroné normal n'est pas un os raccourci mais privé de sa partie distale qui est habituellement annexée par le tibia.

SUMMARY

During normal development of the leg of the chick, growth is competitive between the mesenchyme destined to form the tibia and that destined to form the fibula. The tibia eventually forms a complete bone of large size, using, however, part of the mesenchyme lying on the fibula side. The latter therefore forms only an incomplete bone, no more than two-thirds of the tibial length.

If part of the intermediate mesenchyme, both tibial and peroneal, is excised, competition between the two territories becomes more intense: the tibia develops normally but the fibula does not appear. The material that should have served to make it is monopolized by the tibia alone.

Conversely, if the available mesenchymal material is increased by grafting, the fibula develops well beyond its usual length, attaining that of the tibia, which is unmodified.

The competition can also be suppressed by interposing a sheet of mica between the presumptive territories of tibia and fibula, orientated along the axis of the limb-bud. The mica limits the tibia to material on its side, so that it is a little shorter than usual. The fibula, in contrast, is distinctly longer and its distal extremity unites with the fibulare. The manner of articulation of the heel strongly recalls that of Archaeopteryx.

The competition can also be suppressed by excising and rotating the presumptive fibula through 90°. The tibia and fibula then elongate in contrary directions, and cannot compete with each other: they grow to the same length. In both bones, destruction of cartilage begins in the middle of the diaphysis. This is aberrant, since the normal fibula first ossifies at the distal end of the diaphysis; and it may be considered a proof that the normal fibula is not a shortened bone but a bone deprived of its distal part which has been annexed by the tibia.

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Sur l'induction et la compétence dans les relations entre l'épiblaste et le mésenchyme de la patte de Poulet

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DANS une série d'expériences sur le rôle de l'épiblaste du bourgeon alaire, Saunders (1948) en découpe le bord épaissi et constate un arrêt de la croissance. Les seules ébauches de l'aile qui se développent sont celles qui sont déjà présentes dans le mésenchyme. Les déficiences terminales sont d'autant plus accusées que l'excision est plus précoce, ce qui fait penser que l'épiblaste est responsable, d'une part de la croissance du membre et d'autre part de la détermination successive des divers segments: stylopode (article basal du membre), zeugopode (article intermédiaire) et autopode (article terminal, main ou pied). Mais il était cependant possible d'admettre que le mésenchyme du bourgeon décide par lui-même du segment à produire. Dans cette dernière éventualité, le rôle de l'épiblaste se bornerait à appeler le matériel cellulaire de la base.

INDUCTION ÉPIBLASTIQUE

Dans le but de vérifier l'une ou l'autre des hypothèses précédentes, on sectionne un bourgeon au stade 24 au niveau du genou présumé. La calotte épiblastique est remise en place sur la blessure. Dans 3 cas sur 10, où l'embryon a survécu à l'opération, la patte développée est formée d'un fémur resté en place et se prolongeant par un doigt. La présence de ce doigt résulte de l'activité de l'épiblaste qui coiffait, au moment de l'opération, un mésenchyme de destinée digitale. Il est en tout cas peu probable que le mésenchyme fémoral soit responsable de la formation d'un doigt (Fig. 1A).

Dans une autre expérience de même type, on sectionne un bourgeon au stade 19 au niveau du futur genou. L'épiblaste recouvre à ce stade un mésenchyme presqu'entièrement formé d'éléments du fémur présumé.

La patte développée est constituée d'un fémur basal, de deux tarso-métatarsiens et de deux doigts. Les tibia et péroné sont absents. Comme dans le cas précédent,

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ce sont les éléments terminaux qui se forment, les parties intermédiaires ne régulent pas (Fig. 1B). Dans d'autres cas, la régulation est meilleure, mais dans le cas où elle est imparfaite les pièces terminales se reconstituent en priorité.

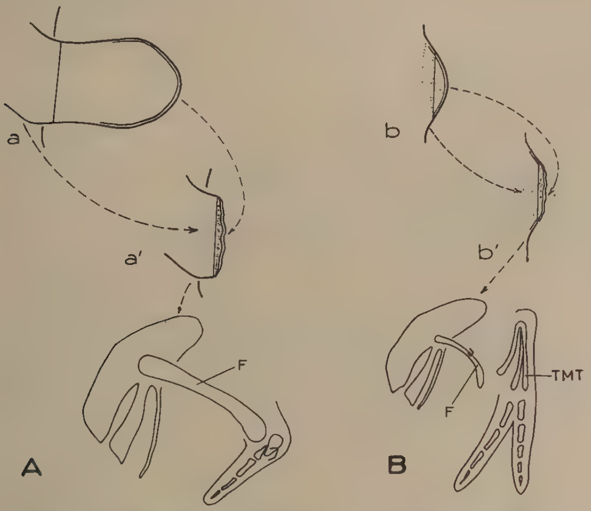

FIG. 1. Rôle inducteur de l'épiblaste. *a*, le bourgeon de patte au stade 24 est sectionné au niveau du genou présomptif. *a'*, la calotte épiblastique est remise en place sur la section. *A*, l'épiblaste a induit le mésenchyme à former un doigt. F, fémur. *b*, bourgeon de patte au stade 19. *b'*, l'épiblaste est remis en place sur la section. *B*, la calotte épiblastique induit la formation des tarso-métatarsiens (*TMT*) et des doigts. F, fémur.

On ne peut expliquer ces résultats qu'en admettant le pouvoir inducteur de l'épiblaste. Celui-ci agirait sur le mésenchyme apical sous-jacent et déterminerait son évolution en éléments qui correspondent à sa phase présente d'induction. L'épiblaste passerait ainsi par des moments d'induction de pièces de plus en plus distales. Grâce à son activité, le mésenchyme terminal serait amené à produire le stylopode pendant la première phase du développement puis, d'autres éléments mésenchymateux se plaçant sous l'épiblaste, une autre vague d'induction les transforme en zeugopode. Si, dans les expériences précédentes, le zeugopode n'est pas remplacé, c'est parce que l'épiblaste ne se trouve plus au stade d'induction de ces pièces.

COMPÉTENCE DU MÉSODERME

Pour assurer un développement correct du membre, il ne faut pas seulement une influence inductrice de la part de l'épiblaste, mais aussi une sensibilité spécifique du mésenchyme.

Afin d'apprécier la variabilité de cette compétence, on appose un épiblaste au stade 22 ou 23 à différents niveaux du bourgeon de membre au stade 22. Les résultats peuvent se résumer comme suit:

(*a*) l'épiblaste implanté dans les flancs de l'embryon n'induit aucune formation osseuse (5 cas);
(*b*) le résultat est le même si l'épiblaste est implanté au niveau de la hanche et du futur fémur (2 cas);
(*c*) l'épiblaste fixé au niveau du futur genou induit la formation d'éléments phalangés (5 cas);
(*d*) implanté sur le tibia proximal, l'épiblaste amène la formation de tarso-métatarsiens et de doigts (3 cas);
(*e*) la patte devient presque complète lorsque l'épiblaste est apposé sur le tibia distal présumé (4 cas).

En suivant une telle série, on ne peut manquer de remarquer la perfection progressive de la patte à mesure que l'épiblaste est apposé plus distalement. Tout se passe comme si le mésenchyme devenait de plus en plus sensible à ses sollicitations. Les résultats sont les meilleurs lorsque le stade d'induction épiblastique correspond à la compétence du mésenchyme.

Aucune réaction ne se remarque lorsque l'épiblaste est implanté dans la base du bourgeon au stade 22. Or, le mésenchyme a dû être sensible à l'action de l'épiblaste au moment où, dans le très jeune bourgeon, il se trouvait en contact avec lui; à ce moment, la calotte épiblastique lui a imposé la destinée fémur. Au stade 22 par contre, le fémur moyen ou basal présumé ne semble pas capable de répondre aux sollicitations de l'épiblaste. Cette insensibilité du mésenchyme est peut-être due au fait que tout le mésenchyme de la base est définitivement affecté au futur fémur, alors que, dans les zones plus distales, beaucoup d'éléments sont affectés au tibia et au péroné, mais à côté subsisteraient d'autres, non touchés par la première induction, ou seulement de façon labile et qui n'attendraient qu'une autre impulsion pour évoluer en tarso-métatarsiens et en doigts.

Cette opinion se confirme par une autre série d'expériences où le bourgeon de patte est régulièrement sectionné au niveau du genou présumé et l'épiblaste, provenant toujours d'un bourgeon au stade 22, remis sur la blessure. Les résultats sont variables et dépendent de l'âge du bourgeon (Fig. 2):

au stade 19, la patte peut être normale;
au stade 20, le tibia est plus court mais l'autopode est encore bien constitué;
au stade suivant, le tibia se réduit au cinquième de sa longueur habituelle, l'autopode est moins réduit;
au stade 22, un doigt fait suite au fémur;
aux stades 23 et 24, l'ébauche fémorale ne peut produire que quelques éléments phalangés. Dans ce dernier cas, l'épiblaste apposé est du même âge que le bourgeon.

Une observation s'impose de prime abord: les parties terminales de la patte s'installent en premier lieu, ce qui confirme le rôle inducteur de l'épiblaste.

D'autre part, les éléments zeugopodiaux qui avaient été excisés ne régulent facilement que sur les bourgeons jeunes. Les bourgeons plus âgés remplacent difficilement ou pas du tout la zone excisée.

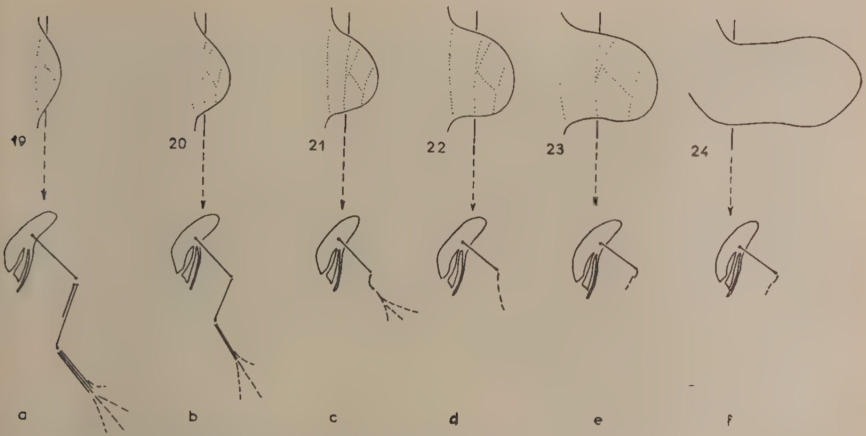

FIG. 2. Tableau récapitulant la réduction de la compétence du mésenchyme. *a*, au stade 19, le mésenchyme fémoral peut fournir suffisamment de matériel pour édifier un zeugopode et un autopode complets. *b*, au stade suivant, le tibia formé est plus court, mais l'autopode est presque normal. *c*, au stade 21, le tibia est réduit au cinquième de sa longueur habituelle; l'autopode est assez bien développé. *d*, l'ébauche fémorale, au stade 22, n'est pas capable d'induire plus d'un doigt. *e*, *f*, à ces stades avancés (23 et 24), l'ébauche fémorale n'est plus capable d'induire que quelques phalanges. Souvent, l'épiblaste ne donne lieu à aucune formation.

Le non-remplacement des éléments excisés du zeugopode pourrait s'expliquer par un appauvrissement progressif de la base en éléments mésenchymateux polyvalents. La base se viderait progressivement de ces cellules qui gagneraient l'apex du bourgeon pour évoluer en éléments osseux plus distaux. L'excision intercalaire aboutit à une réduction des cellules mésenchymateuses disponibles. Celles qui subsistent sont, par priorité, conduites par l'épiblaste vers la formation d'éléments distaux et, s'il y en a suffisamment, elles donneront des pièces zeugopodiales plus ou moins déficientes.

CONCLUSIONS

La croissance et la détermination successive des différents segments du membre d'Oiseau résultent de l'activité inductrice de l'épiblaste qui, au cours du développement du bourgeon, passe par diverses phases d'induction: du stylopode, du zeugopode et de l'autopode. Le mésenchyme du bourgeon est sensible à ces stimuli et répond par la formation d'éléments osseux correspondant à la phase d'induction de l'épiblaste. A mesure que le bourgeon s'accroît, les éléments compétents, c'est-à-dire sensibles, disparaissent de la base et viennent coloniser la zone sous-apicale où l'épiblaste leur imprime leur destinée.

SUMMARY

Epiblastic induction

If the terminal part of the limb-bud of a chick embryo (stage 24) is excised at the level of the future knee, and the exposed surface of the stump is covered with epiblast from the apex of the limb-bud, a toe develops immediately adjacent to the femur. This is explicable if one postulates an inductive power in the epiblast, which before transplantation rested on presumptive digit mesenchyme.

If the epiblast is placed on a stump extending as far as the middle of the presumptive tibia, at stage 19, there form toes and a tarso-metatarsus, joined awkwardly to a tibia deprived of its distal epiphysis. In this case the epiblast induces the formation of toes. The tarso-metatarsus forms imperfectly, probably because the epiblast is no longer fully at the appropriate stage of induction.

Competence of mesenchyme

Placing a cap of epiblast at different levels of the limb-bud produces a range of results. Thus transplantation to the region of the presumptive femur produces no induction. On the other hand, at the level of the presumptive tibia the epiblast induces the formation of phalanges, the more numerous the nearer to the apex.

This decrease from summit to base in the response of the mesenchyme to the epiblast could be due to a progressive diminution of the number of elements not already assigned to a given level and hence still able to participate in forming new or distant segments. It seems that at stage 22 the region of the presumptive femur no longer contains polyvalent elements, these having already been used to construct more distal segments, whence the absence of reaction at this level.

The progressive diminution of polyvalent elements can be tested by sectioning the limb-buds of successively older stages at the level of the future knee and placing on the wound a cap which is at the phase of induction of the toes. After such an operation the terminal parts of the limb develop more readily than the intermediate parts. More important, the young limb-buds regulate more easily (sometimes completely) than the older limb-buds where the piece removed is re-formed with difficulty or even not at all.

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Adenosine Triphosphatase Activity in Early Somite Tissue of the Chick Embryo

by E. M. DEUCHAR[1]

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INTRODUCTION

WE know from the work of Holtzer, Marshall, & Finck (1957), who used a fluorescent antibody staining technique, that by the end of the second day of development in the chick (stage 13, Hamburger & Hamilton, 1951) myosin begins to be identifiable in the myoblast cells of the somites. We have no information so far, however, about the distribution of adenosine triphosphatase in the somites at this early stage, and one of the most characteristic properties of myosin (Engelhardt, 1946) is that it dephosphorylates adenosine triphosphate (ATP), a process which occurs during muscular contraction. The only studies of ATP-ase activity in developing chick muscle (Moog, 1947; Robinson, 1952) have dealt with stages from 12 days' incubation onwards, and have used muscle not all of somitic origin. In an earlier publication Moog & Steinbach (1945) included data on stages as young as 3 days, but used the whole embryo for assays of the enzyme activity. Owing to a lack of adequate micromethods, it was not possible to estimate ATP-ase in somite tissue alone from stages as young as those in which Holtzer *et al.* found myosin antigen.

In the following work a micromethod has been employed to measure ATP-ase activity in somite mesoderm of chick embryos at stage 11 (Hamburger & Hamilton) in which there are approximately equal quantities of segmented and unsegmented mesoderm (Text-fig. 1). The ATP-ase activity of the segmented tissue was compared quantitatively with that of the unsegmented tissue to see whether it was markedly higher in the segmented material, which would suggest that myosin was already being synthesized at this stage. In this study it was necessary to single out an ATP-ase activity that might be attributable to myosin, apart from the ATP-ase carried on the mitochondria of all cells. This was done partly by using different enzyme activators: mitochondrial ATP-ase is activated by magnesium ions, while the ATP-ase most typical of myosin is activated by calcium ions and inhibited by Mg. A differential centrifugation technique was also used to isolate the mitochondria. But the chief problem was, having detected

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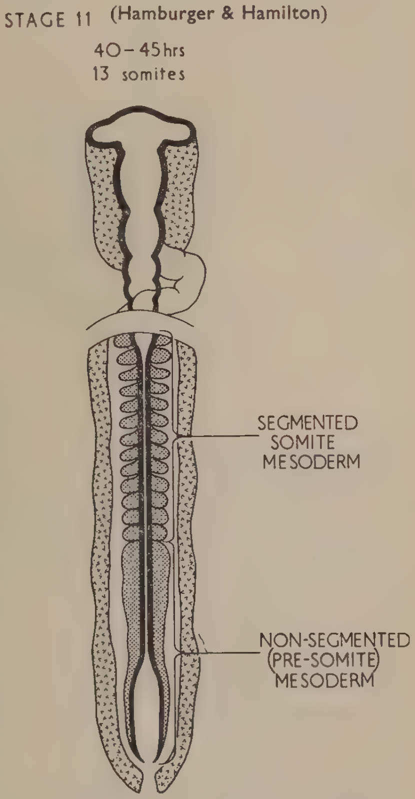
a calcium-activated ATP-ase, to show conclusively that this was bound to myosin or some myosin precursor. A conclusive proof would involve isolating the protein from the tissue. The normal biochemical procedures for isolating myosin were, however, found impracticable for such small quantities of starting material (the complete somites of one embryo had a wet weight of less than 0·5 mg.). So, as a compromise, a glycerol-extraction procedure was used to see whether the ATP-ase was bound to material that was, like myosin, insoluble in glycerol. Holtzer *et al.* found that extraction of chick somites in 50 per cent. glycerol in the cold left the myosin antigen intact in the cells, but apparently leached out the mitochondria and many other intracellular proteins. This same glycerol-extraction procedure has been used to make muscle 'models' which are still contractile and retain their ATP-ase activity (Sarkar, Szent-Györgyi, & Varga, 1950). So in the following work some of the somite tissue was extracted in glycerol before estimating the calcium-activated ATP-ase and compared with control non-extracted tissue.

MATERIAL AND METHODS

Eggs (Rhode Island Red × Light Sussex) were incubated for 45 hours to reach stage 10–11 (Hamburger & Hamilton, 1951). After cutting out the blastoderm and removing the vitelline membrane and yolk from it, the 2 complete strips of somite mesoderm, both segmented and unsegmented (Text-fig. 1), were excised, free of all other tissues except the epidermis which could not be removed without damaging the mesoderm. The operation was carried out in iced Pannett–Compton saline, and the two parts, segmented and unsegmented mesoderm, were collected separately into dishes which were stored in the refrigerator at 2° C. during the time taken to dissect all the embryos (about 6 hours for 20 embryos). The tissues were then transferred via 2 changes of glass-distilled water to micro-homogenizers made of Perspex, and were lightly centrifuged in the cold to concentrate the tissues at the bottom of each tube, so that the supernatant fluid could be pipetted off completely with a micropipette. The tissues were then stored overnight in the homogenizers at —20° C. and the enzyme assays were carried out next day.

ATP-ase assays. After preliminary experiments to determine the optimum pH, incubation time, and concentrations of the reactants, the following procedure was adoped. It was essentially a modification of the method of Lowry & Lopez (1946), adapted to the volumes suitable for a micro-assay. Carlsberg micropipettes (Linderstrøm-Lang & Holter, 1933) were used to pipette accurate volumes of all reagents. The tissues were homogenized in about 25 μl. of ice-cold glass distilled water, and 1 or 2 dilutions of homogenate were made up using distilled water as the diluent. 5·4 μl. samples of homogenate or dilutions were then added to tubes which each contained 5·4 μl. of barbital buffer at pH 9·1, and 1·4 μl. of either 0·05 M $MgCl_2$ or 0·1 M $CaCl_2$ solution. Then 1·4 μl. of 0·1 M ATP (disodium salt: Sigma Products, U.S.A., or Nutritional Biochemicals Company, England), neutralized with N.NaOH, was added to each tube. The tubes were incubated for 1 hour at 25° C., when the reaction was stopped by adding 11 μl. of 15 per cent. trichloracetic acid followed by 100 μl. of 0·1 N sodium acetate to raise the pH to 4. After centrifuging for 15 minutes at 5,000 r.p.m., 100 μl. of each supernatant was transferred to a second tube and a further 100 μl. of acetate buffer at pH 4 (Lowry & Lopez, 1946) added to it. The extinction at 700 mμ was read, using microcells, on a Hilger Uvispek Spectrophotometer 20 minutes after adding the reagents for phosphate determination (22 μl. each, of 1 per cent. ascorbic acid and 1 per cent. ammonium molybdate). The ammonium molybdate was made up freshly before each set of determinations. The extinction values, calibrated by means of a standard curve obtained with known concentrations of potassium phosphate solution, gave a measure of the total inorganic phosphate per sample. Homogenate blanks (distilled water instead of ATP) and reagent blanks (distilled water instead of homogenate) were run with each experiment, and were subtracted from the experimental values to give the total phosphate liberated from ATP by ATP-ase. 5·4-μl. samples of the homogenates were assayed for total nitrogen by the ultramicro-kjeldahl method of Boell & Shen (1954). Results were then expressed as γP liberated per hour per γ total nitrogen in the samples.

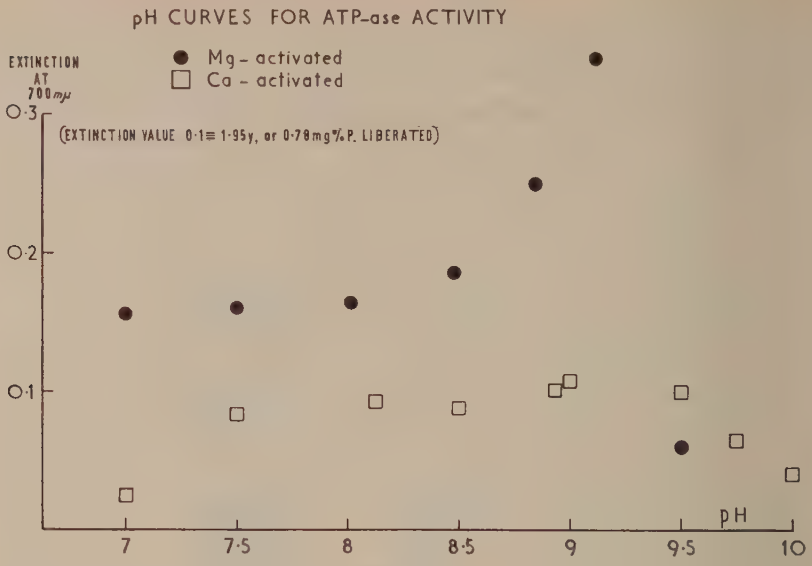

STAGE 11 (Hamburger & Hamilton)
40–45 hrs
13 somites
SEGMENTED SOMITE MESODERM
NON-SEGMENTED (PRE-SOMITE) MESODERM

TEXT-FIG. 1. Diagram of chick embryo showing areas excised for ATP-ase assay.

EXPERIMENTAL RESULTS

Characterization of ATP-ase activity

In separate series of experiments the ATP-ase determinations were carried out using either magnesium or calcium chloride as activators. As the graphs of Text-fig. 2 show, there was a much higher activity in the presence of magnesium than in the presence of calcium ions, and both these activities showed maxima, more marked for Mg-activated than for Ca-activated ATP-ase, at pH 9. Both activities were present in unsegmented as well as in segmented mesoderm.



TEXT-FIG. 2

Effect of glycerol-extraction on ATP-ase activity

Somite mesoderm, collected from about 40 embryos, was divided into two approximately equal-sized samples, one of which (control) was stored in the deep freeze after removal of the distilled water used to wash it, in the usual way, while the other was transferred to 50 per cent. glycerol (buffered with 0·02 per cent. sodium bicarbonate) before storing it in the deep freeze overnight. Next day the glycerol was removed and replaced with two successive changes of chilled glass-distilled water, centrifuging lightly in the cold each time in order to concentrate the tissue and to be able to pipette off all the fluid from above it. The control tissue was subjected to the same centrifuging as the glycerol-extracted tissue, but without further washing in distilled water. Both Ca- and Mg-activated ATP-ase activity were then compared between glycerol-extracted and control tissue. As Table 1 shows, the Mg-activated ATP-ase per unit of total nitrogen was markedly lower in the glycerol-extracted tissue than in the control tissue, but, in contrast, the Ca-activated ATP-ase per unit of total nitrogen was significantly *higher* ($t = 2{\cdot}86$ for 7 d.f.; $P < 0{\cdot}05$, testing the differences in the 5 experiments). There was, of course, a decrease of total nitrogen in the tissue after it had been extracted in glycerol, and these results indicate that the nitrogenous material extracted by the glycerol included magnesium-activated, but not calcium-activated, ATP-ase.

Preparation of mitochondrial fraction

Somite tissue which had been collected in the usual way was transferred to 6 per cent. sucrose solution (buffered to pH 7 with 0·2 g./l. sodium bicarbonate).

It was homogenized in approximately 200 μl. of this, and centrifuged for 10 minutes at 3,500 r.p.m. in a 'Misco' microcentrifuge at refrigerator temperature. The precipitate *P* was washed, after conserving the supernatant, and was recentrifuged in the same way; the washings were then discarded. The supernatant was now centrifuged for 15 minutes at 21,000 r.p.m. (=24,000 g.) to obtain a precipitate of mitochondria *M*. The final supernatant *S* contained only very sparse mitochondria. A sample of each fraction was looked at under phase contrast to give some idea of how good a separation of the mitochondria from other cell constituents had been achieved.

TABLE 1

Effects of glycerol-extraction on ATP-ase activity

Data expressed as γ phosphorus liberated per hour per γ nitrogen in tissue

Note: In this and the following tables, each figure is the mean of 2 readings obtained with a homogenate of tissue from at least 12 embryos. Where there are more than 1 figure in an experiment, different dilutions of the same homogenate have been used.

(*a*) *Magnesium-activated ATP-ase*

Experiment	*Controls*	*Glycerol-extracted*	*Difference*
1	0·571	0·393	0·178
2	0·914	0·617	0·297
	1·022	0·699	0·323
3	0·486	0·301	0·185
	0·503	0·312	0·191
Mean	0·675	0·462	0·235
Standard error	±0·113	±0·082	±0·031

(*b*) *Calcium-activated ATP-ase*

Experiment	*Controls*	*Glycerol-extracted*	*Difference*
1	0·0429	0·0837	0·0308
	0·0438	0·0933	0·0495
2	0·0336	0·0540	0·0204
3	0·0504	0·0700	0·0196
	0·0504	0·0637	0·0133
4	0·0519	0·0689	0·0170
	0·0564	0·0705	0·0141
5	0·0769	0·1708	0·0959
Mean	0·0508	0·0803	0·0323
Standard error	±0·004	±0·012	±0·009

ATP-ase activity in mitochondrial fraction. Mg-activated ATP-ase activity per unit total nitrogen was estimated in the first precipitate *P*, the mitochondrial fraction *M*, and the supernatant *S*. The results (Table 2*a*) show three times as much Mg-activated ATP-ase activity in the mitochondrial fraction *M* as in the first precipitate *P*. At least some of the activity present in the other fractions may be attributed to contamination with mitochondria, since phase-contrast observations showed some mitochondria in both these fractions. The Ca-activated ATP-ase, on the other hand, is localized chiefly in *P* (Table 2*b*).

TABLE 2

Distribution of ATP-ase activity in centrifugal fractions

Data expressed as γ phosphorus liberated per hour per γ nitrogen in sample

(*a*) *Magnesium-activated ATP-ase*

	Precipitate (*P*)	*Mitochondria* (*M*)	*Supernatant* (*S*)
Expt. 1 . .	0·1186	0·3386	0·0455
Expt. 2 . .	0·0792	0·2093	None detectable

(*b*) *Calcium-activated ATP-ase*

	Precipitate (*P*)	*Mitochondria* (*M*)	*Supernatant* (*S*)
Expt. 1 . .	0·142	0·087	0·053
Expt. 2 . .	0·206	0·099	0·028

Differences in ATP-ase activity between segmented and non-segmented somite mesoderm

When samples of these two portions of tissue were compared, it was found that they did not differ significantly in Mg-activated ATP-ase activity, but that the Ca-activated ATP-ase per unit total nitrogen was significantly higher in segmented somite tissue than in the non-segmented somite tissue ($t = 6{\cdot}75$ for 9 d.f.; $P < 0{\cdot}01$ for the differences in Table 3).

TABLE 3

Calcium-activated ATP-ase activities in segmented and unsegmented somite mesoderm

γ phosphorus liberated per hour per γ nitrogen

	Segmented	*Unsegmented*	*Difference*
Expt. 1 . .	0·0769 0·0578 0·0512	0·0481 0·0307 0·0338	0·0288 0·0271 0·0174
Expt. 2 . .	0·1134 0·1137 0·1187	0·0811 0·0817 0·0724	0·0302 0·0297 0·0395
Expt. 3 . .	0·0862 0·0901 0·0800	0·0751 0·0738 0·0738	0·0111 0·0163 0·0062
Mean . .	0·0876	0·0634	0·0229
Standard error .	±0·008	±0·007	±0·004

DISCUSSION

The comparisons between segmented and non-segmented somite mesoderm described above indicate that there is a definite, though small, increase in calcium-activated ATP-ase per unit of nitrogen in the tissue that has just segmented. The fact that the magnesium-activated (mitochondrial) ATP-ase shows no regional difference at this time, emphasizes that the calcium-activated ATP-ase may have some specific role that is not the same as that of the general cellular ATP-ase. The finding that the Ca-activated ATP-ase is insoluble in glycerol lends weight to the idea that this enzyme activity is bound to a material resembling myosin. But Holtzer, Marshall, & Finck (1957) were not able, even by their highly sensitive method, to detect myosin in a form capable of reacting with antibody until a later stage than this (stage 13: 48–52 hours). It would seem, then, either that the ATP-ase represents some precursor state not yet with the molecular structure capable of giving the antigen reaction of myosin, or that the myosin is not yet highly concentrated enough in myoblast cells to be detectable by immuno-histochemical means.

SUMMARY

1. Segmented somite tissue of 40 to 45-hour chick embryos (stage 11, Hamburger & Hamilton, 1951) shows a higher calcium-activated ATP-ase activity than the somite tissue that has not yet segmented at this stage. The enzyme activity is not extracted by prior treatment of the tissues with 50 per cent. glycerol, and is not localized in the mitochondrial fraction.

2. There is no difference in magnesium-activated (mitochondrial) ATP-ase activity between segmented and non-segmented mesoderm at this stage.

3. It is postulated that the apparent increase in calcium-activated ATP-ase at the time of segmentation may represent a synthesis of myosin or of some myosin precursor.

RÉSUMÉ

L'activité de l'adénosine triphosphatase sur le tissu somitique jeune de l'embryon de Poulet

Le tissu somitique segmenté de l'embryon de Poulet de 40 à 45 heures (stade 11, Hamburger & Hamilton, 1951) montre une activité plus intense de l'ATP-ase, activée par le Calcium, que le tissu somitique qui n'est pas encore segmenté à ce stade. L'enzyme actif n'est pas extrait par un traitement préalable avec 50 pour cent de glycérol. Il n'y a pas de différence dans l'activité de l'ATP-ase activée par le Magnésium entre le mésoderme segmenté et le mésoderme insegmenté à ce stade. L'auteur émet l'hypothèse suivant laquelle l'augmentation apparente de l'ATP-ase activée par le Calcium au moment de la segmentation des somites pourrait correspondre à une synthèse de myosine ou d'un précurseur de la myosine.

ACKNOWLEDGEMENTS

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Relation between Somite Segregation Rate and ATP-ase Activity in Early Chick Embryos

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In the preceding paper (Deuchar, 1960) it was established that the first-formed somites of the chick embryos at 40–45 hours' incubation (Hamburger & Hamilton's stage 11, 1951) have a higher calcium-activated ATP-ase activity per unit nitrogen than the somite mesoderm which is not yet segmented at this stage. It was inferred that there is an increase of ATP-ase (possibly connected with myosin synthesis) in the somite tissue as it segments. A question that at once arises is how far the ATP-ase activity is essential to the process of somite segmentation. Besides presaging myosin, the ATP-ase could conceivably play an immediate role during the movements and changes of shape that the mesoderm cells undergo at this time. If so, one would expect some correlation between intensity of ATP-ase activity and the efficiency of segmentation, if either were modified experimentally. To test this possibility, in the work to be described here chick embryos have been grown in media to which the agents ω-bromoallylglycine, leucine, and adenosine triphosphate had been added. ω-bromoallylglycine (BAG) is an analogue of leucine which was found by Herrmann, Konigsberg, & Curry (1955) to block completely the segmentation of chick somites. Its effects are reversible by the simultaneous addition of leucine. In the present experiments its effects on ATP-ase activity as well as segmentation rate were measured, both alone and in the presence of leucine. Leucine was also tested by itself to see if it would increase either segmentation rate or ATP-ase activity above the normal level. Finally, ATP was added to some cultures as a possible means of raising both ATP-ase activity and segmentation rate. ATP was then used in combination with BAG to see if it would reverse the effects of this inhibitor.

In all experimental series, chick embryos of 32 hours' incubation were cultured for 10 hours in an agar medium containing the modifying agent. The number of somites formed in the 10 hours by each embryo was recorded, and the somite tissue was then dissected out to be used for assays of ATP-ase activity.

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MATERIAL AND METHODS

Chick blastoderms were removed from the eggs at 32 hours' incubation (stage 8–9 of Hamburger & Hamilton's series), and were trimmed just outside the area vasculosa so that its boundary (future sinus terminalis) was included intact. The vitelline membrane and all adhering yolk were removed and the blastoderms were then explanted, ectoderm downwards, on to clots of agar medium in watch-glass cultures. The *medium* was made up as follows: Pannett–Compton saline, at double the normal concentration and containing 2 per cent. glucose, was autoclaved and then buffered (when cool) by adding 0·04 g. per cent. sodium bircarbonate. This saline was mixed 1:1 by volume with a 1·5 per cent. aqueous solution of agar that had previously been boiled then cooled to about 40° C. The mixture was poured into sterile watch-glasses in culture dishes and allowed to set. The reagents used in the various experimental series were dissolved in the saline immediately before it was mixed with agar. Wash solutions were made by mixing aliquots of saline with an equal volume of distilled water, and before being transferred to a clot containing a given reagent each embryo was taken through a wash in saline containing the same concentration of the reagent.

Reagents

The ω-bromo-allylglycine (BAG) was stored at —20° C. and used at concentrations of 0·15 and 0·30 mg./ml. of culture medium. The DL-leucine (B.D.H. Laboratories Ltd.) was ground to a powder to make it more easily soluble, and was used at concentrations of 0·3 and 0·4 mg./ml. of culture medium. Adenosine triphosphate (the disodium salt, Nutritional Biochemical Laboratories Ltd.) was weighed out immediately before use, and after dissolving it in the saline this solution was at once neutralized with a few drops of N.NaOH. The final concentration of ATP in the culture medium was 0·008 M.

After the embryos had been cultured for 10 hours at 38° C., the number of somites formed was counted in each embryo and the somite tissue, both segmented and unsegmented, was dissected out from at least twelve embryos and collected in ice-cold saline. After being washed in two changes of ice-cold distilled water it was transferred in this fluid to a Perspex micro-homogenizer. After homogenizing, and still in the cold, the micro-homogenizer was centrifuged slightly to precipitate the tissue and the supernatant was then carefully pipetted off. The tissue was stored in the deep freeze overnight. Next day, ATP-ase assays were carried out as described in the previous paper (Deuchar, 1960) by a micro-adaptation of the method of Lowry & Lopez (1946).

Statistical analysis

With the data on somite numbers, comparison of variances between and within experiments showed the ratio F to be insignificant, so that the data in each group of experiments could be pooled and tested as a whole by a *t*-test for significant differences between experimental and control series. With the ATP-ase activity data, *t*-tests were carried out on differences between the values for experimental and control embryos. The standard errors of these differences, as well as the standard errors of each group of data, are quoted in the tables.

RESULTS

Treatment with BAG

At a concentration of 0·15 mg./ml., BAG did not completely block the segmentation of somites in the embryos and did not significantly reduce the number

TABLE 1 (*a*)

Effect of BAG on somite segmentation

Mean numbers of somite pairs formed per embryo in 10 hours
Data from a total of 87 control and 89 treated embryos

Experiment	*Controls*	*BAG-treated*
1	7·90	7·44
2	6·31	5·67
3	6·80	6·20
4	6·40	5·90
5	5·90	5·75
6	6·62	5·66
7	4·80	3·75
8	6·12	5·00

TABLE 1 (*b*)

Effect of BAG on calcium-activated ATP-ase

Data expressed as γ P liberated per hour per γ nitrogen in tissue sample. In this and the following tables, each figure for ATP-ase activity is a mean of two readings obtained with a homogenate of tissue from at least twelve embryos. Where there are two figures for one experiment, these are obtained at two different dilutions of the same homogenate

Experiment	*Controls*	*BAG-treated*
1	0·105 0·109	0·074 0·074
2	0·242 0·217	0·179 0·156
3	0·189 0·207	0·149 0·139
4	0·242 0·266	0·179 0·181
5	0·160 0·139	0·099 0·098
Mean	0·188	0·133
Standard error	±0·018	±0·014

Mean and standard error of differences between BAG-treated and controls = 0·055 ± 0·005.

of somites formed in 10 hours' culture (Table 1 (*a*): $t = 1·70$, d.f. = 175). However, the ATP-ase activity of somites in the treated embryos was significantly lower than in the controls which were grown in agar-saline without BAG (Table 1 (*b*): $t = 8·12$, d.f. = 4, $P < 0·001$).

At a concentration of 0·30 mg./ml., BAG greatly reduced the number of somites formed in 10 hours (data of Tables 2 (*a*) and 5 (*a*) pooled: $t = 8·87$, d.f. = 106, $P < 0·001$). Some embryos even showed *fewer* somite pairs at the end of the culture period than they had initially, indicating that some of the already existing somite blocks had disintegrated or de-differentiated. The ATP-ase activity was, again, lower in the treated embryos than in controls, but this difference was not significant according to the few and rather variable data available (data of Tables 2 (*b*) and 5 (*b*) pooled: $t = 2·31$, d.f. = 4, $0·05 < P < 0·1$).

Reversal of BAG with leucine

Following Herrmann, Konigsberg, & Curry's (1955) procedure, 0·3 mg./ml. leucine was added to media containing 0·3 mg./ml. of BAG. As can be seen from Table 2 (*a*), the addition of leucine completely restored the process of segmentation ($t = 7·83$, d.f. = 33, $P < 0·001$). The ATP-ase activity was also significantly higher in the leucine-and-BAG-treated embryos than in those treated with BAG alone (Table 2 (*b*): $t = 4·169$, d.f. = 3, $P < 0·05$).

TABLE 2

(*a*) *Reversal by leucine of BAG effect on somite segmentation*

Mean numbers of somite pairs formed per embryo in 10 hours. Data from total of 17 control, 36 (BAG and Leucine)-treated, and 32 BAG-treated embryos

Experiment	*Controls*	*BAG*	*BAG+leucine*
1	—	0·0	5·94
2	4·83	0·625	5·45
3	5·86	0·625	6·67

(*b*) *Reversal by leucine of BAG effect on ATP-ase activity*

Expressed as γ phosphorus liberated per hour per γ nitrogen in sample

Experiment	*Controls*	*BAG*	*BAG+leucine*
1	—	0·434	0·475
2	0·121	0·114	0·143
3	0·250	0·220	0·316
4	—	0·441	0·527
Mean	0·185	0·302	0·365
Standard errors	±0·064	±0·081	±0·068

Mean difference between BAG and control series = 0·019±0·011.
Mean difference between BAG and (BAG+leucine) series = 0·063±0·016.

Effect of leucine alone

The leucine was added to the agar medium in a concentration of 0·4 mg./ml. After 10 hours' culture the leucine-treated embryos had not formed significantly more somite pairs than control embryos, nor was the ATP-ase activity of their somite tissue any higher than in the control tissue (Table 3).

TABLE 3

(*a*) *Effect of leucine alone on somite segmentation*

Mean numbers of somite pairs formed per embryo in 10 hours
Data from a total of 30 control and 30 treated embryos

Experiment	*Controls*	*+Leucine*
1	4·31	5·25
2	5·86	6·89
3	7·00	8·31

(*b*) *Effect of leucine alone on ATP-ase activity*

γ phosphorus liberated per hour per γ nitrogen in sample

Experiment	*Controls*	*+Leucine*
1	0·155	0·403
2	0·250	0·374
3	0·177	0·212
4	0·228	0·488
Mean	0·203	0·369
Standard Error	±0·022	±0·057

Mean and standard error of differences between treated and control embryos = 0·167 ±0·054.

Treatment with ATP

Since in a preliminary experiment a high mortality had occurred among embryos treated with ATP concentrations higher than 0·008 M, this concentration was used in the following experiments, which generally had a 100 per cent. survival rate. As Table 4 (*a*) shows, the embryos grown in medium containing 0·008 M ATP formed significantly more somites than the control embryos ($t = 3{\cdot}77$, d.f. = 83, $P < 0{\cdot}001$). Their somite tissue also had a significantly higher ATP-ase activity than the controls' tissue (Table 4 (*b*): $t = 6{\cdot}73$, d.f. = 8, $P < 0{\cdot}001$).

TABLE 4

(*a*) *Effect of ATP on somite segmentation*

Mean numbers of somite pairs formed per embryo in 10 hours
Data from a total of 43 control and 42 treated embryos

Experiment	*Controls*	*+ATP*
1	5·83	8·75
2	5·87	6·57
3	6·30	7·30
4	5·16	6·83
5	5·36	7·00

(*b*) *Effect of ATP on ATP-ase activity*

γ phosphorus liberated per hour per γ nitrogen in sample

Experiment	*Controls*	*+ATP*
1	0·074	0·112
2	0·257	0·340
3	0·138 0·149	0·181 0·180
4	0·051	0·085
5	0·117 0·093	0·219 0·181
6	0·120 0·099	0·180 0·148
Mean	0·122	0·181
Standard error	±0·019	±0·024

Mean and Standard error of differences between treated and control embryos = 0·059 ± 0·009.

Reversal of BAG effects with ATP

0·008 M ATP was added to medium already containing 0·3 mg./ml. BAG, and embryos were cultured on this, together with a set of embryos on medium containing BAG alone, and a control set on pure agar-saline. In the presence of ATP, more somites were formed than in BAG-treated embryos without ATP. In experiments 2 and 3 of Table 5 (*a*), these differences were significant ($t = 2{\cdot}11$, d.f. = 26 in expt. 2, $P < 0{\cdot}005$; $t = 2{\cdot}96$, d.f. = 24 in expt. 3, $P < 0{\cdot}01$). But

TABLE 5

(*a*) *Reversal by ATP of BAG effects on somite segmentation*

Mean numbers of somite pairs formed per embryo in 10 hours
Data from a total of 39 BAG-treated, 36 (BAG + ATP)-treated, and 36 control embryos

Experiment	*Controls*	*BAG*	*BAG+ATP*
1	6·10	3·58	3·77
2	6·40	1·30	3·00
3	5·31	2·36	4·96

(*b*) *Reversal by ATP of BAG effects on ATP-ase*

γ phosphorus liberated per hour per γ nitrogen in sample

Experiment	*Controls*	*BAG*	*BAG+ATP*
1	0·259	0·118	0·156
2	0·107	0·086	0·115
3	0·272	0·170	0·252
Mean	0·213	0·125	0·174
Standard error	±0·053	±0·024	±0·028

Mean difference between BAG-treated and controls = 0·088 ± 0·032.
Mean difference between BAG and (BAG + ATP) series = 0·050 ± 0·016.

there were still not as many somites formed in the embryos treated with BAG + ATP as in controls: these differences were significant in experiments 1 and 2 ($t = 3{\cdot}48$, d.f. = 10 in expt. 1, $P < 0{\cdot}01$; $t = 4{\cdot}93$, d.f. = 25 in expt. 2, $P < 0{\cdot}001$). The ATP-ase activity appeared to be slightly restored by addition of ATP with the BAG (see Table 5 (*b*)), but according to these few data the differences were not statistically significant. ATP did not, therefore, reverse the effects of BAG as clearly as did leucine.

DISCUSSION

The results of these experiments are consistent with the idea that calcium-activated ATP-ase activity and the process of somite segmentation are in some way interdependent. Whenever the normal segmentation rate was modified—increased or decreased—there was a corresponding change in ATP-ase activity in the somite tissue. Particularly cogent are the experiments in which the action of BAG was reversed, and this restored not only the segmentation rate but also the ATP-ase activity. This suggests very strongly that the two processes are closely linked.

We know from the work of Hoffmann-Berling (1956) that contractile systems in a number of different animal cells respond to ATP and are accompanied by ATP breakdown. Now, as the somites form in Vertebrate embryos, the originally cuboid mesoderm cells become more fusiform and re-associate in concentric groups. It may well be that they carry out these movements with the help of ATP-breakdown, too. If so, it is to be expected that adding ATP would assist the segmentation (as it apparently does in these experiments) by providing more substrate for ATP-ase action and possibly even inducing the synthesis of more ATP-ase. It could be for the same reason that ATP is able to some extent to counteract the inhibition by BAG. But it is not very clear why BAG should have any direct effect on ATP-ase activity. We know from the work of Schultz & Herrmann (1958) that BAG inhibits protein synthesis in the somites, presumably by competing with leucine, of which it is an analogue biochemically. One must assume, then, that ATP-ase, and perhaps hence myosin, are not synthesized at the normal rate in the presence of BAG. It is easy to see that this situation should be restored, as indeed it was in the foregoing experiments, by adding the particular amino-acid that is blocked by BAG, namely, leucine.

It should be possible to throw more light on the exact function of ATP-ase during somite segmentation by examining the effect of these same agents, BAG, leucine, and ATP, as well as ATP-ase inhibitors, on myoblast cells or fragments of somite mesoderm in tissue culture.

SUMMARY

1. When chick embryos of 32 hours' incubation are grown on an agar medium containing the leucine antimetabolite ω-bromo-allylglycine (BAG) which inhibits somite segmentation (Herrmann, Konigsberg, & Curry, 1955), the calcium-

activated ATP-ase activity of the somite mesoderm is lower than in untreated controls grown on agar alone.

2. Addition of leucine simultaneously with BAG reverses both its effect on somite segmentation and its effect on ATP-ase.

3. Leucine added alone to the medium, without BAG, increases both the rate of segmentation and the ATP-ase activity of the somite tissue.

4. 0·008 M ATP added to the medium also increases the rate of somite segmentation, and the ATP-ase activity is higher in this case too.

5. 0·008 M ATP is able partially to reverse the effects of BAG, but not so efficiently as leucine.

6. It is postulated that the movements and shape changes of the paraxial mesoderm cells during initial somite formation may depend, like many contractile systems in animal cells, on ATP-ase activity. The ATP-ase activity in some of these particular cells (myoblasts) may also presage myosin synthesis.

RÉSUMÉ

Sur la relation entre la segmentation des somites et l'activité de l'ATP-ase chez les jeunes embryons de Poulet

Quand des embryons de Poulet de 32 heures d'incubation sont explantés sur un milieu gélosé contenant l'antimétabolite de la leucine, la ω-bromo-allylglycine (BAG), qui inhibe la segmentation des somites (Herrmann, Konigsberg et Curry, 1955), l'activité de l'ATP-ase, activée par le calcium, du mésoderme somitique, est plus réduite que celle de témoins non traités cultivés sur agar seul. L'addition de leucine en même temps que de BAG inverse son action sur la segmentation des somites et sur l'ATP-ase.

La leucine ajoutée seule au milieu, sans BAG, augmente à la fois le rythme de segmentation et l'activité de l'ATP-ase du tissu somitique.

L'addition de 0,008 M d'ATP augmente aussi le rythme de la segmentation des somites, et l'activité ATP-asique est aussi plus élevée dans ce cas. Les effets du BAG peuvent être partiellement inversés par l'apport de 0,008 M d'ATP, mais pas avec autant d'efficacité que par la leucine.

On suppose que les mouvements et les changements de forme du mésoderme paraxial au début de la formation des somites peut dépendre de l'activité ATP-asique, comme c'est le cas dans beaucoup de systèmes contractiles des cellules animales. Dans certaines de ces cellules spéciales (myoblastes), l'activité ATP-asique peut aussi présager la synthèse de myosine.

ACKNOWLEDGEMENTS

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The Sexual Development of *Salpa fusiformis* (Cuvier)

Part I

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From the Chelsea College of Science and Technology

INTRODUCTION

In 1928 Brien wrote: 'L'embryogénèse des Salpes quoique étudiée depuis longtemps, n'en est pas moins restée relativement obscure. Les interprétations les plus diverses, souvent contradictoires, ont été émises.' The position has been altered but little by Brien's own work and by that of Berrill (1950) which merely claimed to have confirmed the observations made by Brooks (1893).

The major discrepancies in the literature are summarized in the Table and can, without doubt, be attributed to two causes.

The first is the peculiar nature of salp development. At an early stage the blastomeres become separated from each other by cells proliferated from the wall of the follicle in which the embryo begins its development. It is necessary for the later aggregation of the blastomeres to form the definitive embryo, that there should be spaces through and around which the blastomeres can move, a factor of salp development hitherto unrecognized. Later definitive body cavities also appear within the developing oozooid. It has been found in the course of the present investigation that spaces formed by the degeneration of follicle cells provide blastomere migration routeways and that some of these spaces also become incorporated into the body cavities of the developing oozooid. Thus two types of cavity are produced as the result of follicle cell degeneration, those appearing at random—i.e. rarely in the same positions in any two embryos—and those which develop in almost identical positions in all embryos and which later become incorporated into the body cavities of the developing oozooids. Considerable confusion in the identification of the two types of cavity is evident in the earlier accounts and almost certainly stems from the examination of too small a number of specimens. The examination of large numbers is essential. At no stage are any two specimens exactly alike, although all have certain features in common. To distinguish the significant from the relatively insignificant, e.g. in the nature of the future placental cavities, or the blastomere arrangement,

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many animals must be examined at each stage. Berrill (1950) alone of all the authorities cited the number investigated. They range from a minimum of five of *Salpa maxima* to 47 of *Thalia democratica*. It seems possible that other authorities may similarly have examined only small numbers of specimens at many of the stages described.

The second cause for confusion in the literature is a lack of material at certain critical stages of development. Thus, the earliest embryo examined by Korotneff (1896*b*) was at the eight-cell stage; he, therefore, did not see the initiation of the immigration of the follicle cells ('calymmocytes', Salensky, 1876) between the blastomeres and so incorrectly identified the migrating calymmocytes as micromeres, a criticism which he himself applied to Heider (1895): '. . . der Unterschied zwischen Blastomeren und Kalymmocyten von Heider nicht genügend erkannt ist.' Both Korotneff and Heider confess to a lack of material; Korotneff came to the conclusion that the missing stages were 'nicht so besonders wichtig'; Heider, more circumspect, admitted that his material was limited and that, therefore, the conclusions to which he came, in particular that concerning the provenance of the cells forming the definitive oozooid, required further proof. The present investigation has shown that Brien (1928) and Brooks (1893) would seem similarly to have lacked material of a critical nature.

It was decided, therefore, that a re-investigation of the sexual development of a thecogone and of a gymnogone salp, *S. fusiformis* Cuvier and *T. democratica* Forskal, respectively, was desirable. The present work is an account of the early development of *S. fusiformis*. In its course more than 200 specimens were sectioned and an additional forty-two were subjected to tests while living or when freshly killed. Over 100 more have been examined for the later development, but there are still several stages lacking, and thus this part of the work is yet incomplete. It is hoped that material recently received and promised will provide at least some if not all these stages, and that the account of the embryogenesis of *S. fusiformis* may soon be completed.

MATERIAL AND METHODS

The material for the present work was collected from the Atlantic Ocean (47° 20′ N., 6° 23′ W.) in May 1957, from the Straits of Messina in April 1955 and 1956, and a very small number was obtained from the Bay of Palermo in April 1956. Tests upon freshly killed or living material were performed at Palermo and Messina. The youngest blastozooids were obtained from the Atlantic Ocean, those rather older from all three sources.

Specimens to be sectioned were preserved in Helly Zenker, aqueous Bouin, or in Du Boscq Brasil. From the great majority serial sections were cut at 2 μ and stained in Heidenhain's iron haematoxylin and counterstained with light green. The Feulgen test was applied to appropriate sections, and where positive results were confirmed by the use of Unna-Pappenheim (Brachet's technique, 1953) on identical sections, it has been assumed that desoxyribose nucleic acid

(DNA) was present. The distribution of glycogen was demonstrated by means of Best's carmine stain.

Fresh material was vitally stained with neutral red, Janus green B, or was subjected to Mancuso's test for glutathione (1952).

OBSERVATIONS

There are two periods in the early development of *S. fusiformis*: (i) development within the follicle ('uterine sac', Brien, 1928) prior to the rupture of the atrial wall; and (ii) development of the blastophore from the rupture of the atrial wall to the period immediately after closure of the incubation fold.

The development of the follicle prior to the rupture of the atrial wall

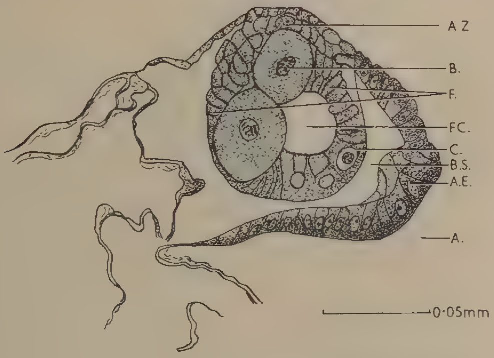
Sawicki (1958) has described the development of the unfertilized egg in the chain salp. Unfortunately it has not been possible to obtain blastozooids in which the fertilized pre-cleavage egg is present, and thus the youngest embryo investigated in the course of the present work was at the two-cell stage of development.

By the time two blastomeres are to be observed within the follicle the latter has reached its definitive position dorso-laterally and to the right, immediately posterior to the sixth muscle-band of the parent blastozooid. The sac lies close to the epithelium of a low fold of the atrial wall which, as a result, projects into the cloaca at this point. In this region the typical pavement epithelium lining the atrium becomes cubical or columnar. The follicle is bathed by the blood in a maternal blood-sinus.

The sac is round and has a diameter of 0·08 mm. and within it, adjacent to the wall on one side, lie two relatively large contiguous blastomeres, bathed on their free surfaces by the follicular fluid. They are ovoid and slightly basophil. The cytoplasm contains granules of varying sizes, but there are few mitochondria. The nucleus is relatively small (Text-fig. 1).

The second and third cleavages are normal. At the early eight-cell stage the blastomeres form a morula (Text-fig. 2) attached to the follicle wall by a small group of cells proliferated by the follicular epithelium. The blastomeres differ slightly in appearance from each other. For example, in one of my preparations those close to the attachment zone are flattened and the remainder round; while in others, although no such marked difference can be observed, the cells vary somewhat in shape. There is, however, no regularity in the arrangement and it is not possible to distinguish micro- from macromeres as Heider (1895) and Korotneff (1896*b*) suggest. The variations in shape are undoubtedly due to compression by neighbouring blastomeres.

During the eight-cell stage the first of the anomalies in salp development occurs. The follicle cells proliferate rapidly and the cells so produced insinuate themselves between the blastomeres in such a manner as to cover the morula completely and to enclose each blastomere in a single-layered sheath of flattened cells. Concurrently each blastomere becomes almost completely separated from its sheath by a space. At one point alone are sheath and blastomere in contact (Text-fig. 3). Thus, while no evident blastula is formed, it is possible that the spaces separating the blastomeres from their sheaths represent an incipient blastocoel. The 'micromeres' described by Heider (1895) and Korotneff (1899) are beyond doubt immigrating calymmocytes, a lack of the very young stages in which the calymmocyte invasion is initiated evidently being responsible for the confusion. It is significant that Heider admitted to a great difficulty in distinguishing follicle cells from 'micromeres'. The separation of the blastomeres from each other is followed by a process whose significance has been much disputed (see the Table). Each blastomere fragments into five or, typically, seven small 'massifs' and one larger portion containing a large nucleus. These 'massifs'



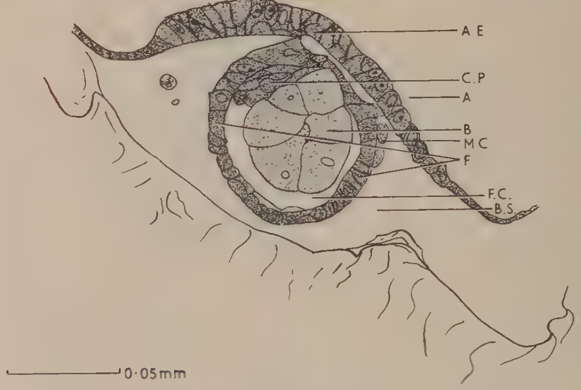
TEXT-FIG. 1. *S. fusiformis*: section through the follicle at the two-cell stage.

Key to the text-figures

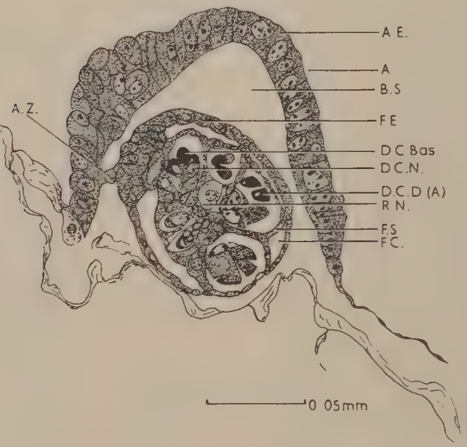
All text-figures were drawn with the aid of a camera lucida.

A.	Atrium (cloaca).
A.E.	Atrial epithelium.
A.Z.	Attachment zone of follicle to atrial epithelium.
B.	Blastomere.
Bl.	Blastophore.
Bl.C.	Cavity within the blastophore.
Bl.–A.J.	Junction between the blastophore and the atrial epithelium.
Bl.E.	Epithelium of blastophore.
B.(N-C).	Neuro-chordal blastomeres.
B.S.	Maternal blood sinus.
C.	Calymmocyte (follicle cell).
C.B.	Ectodermal blastomere, forerunner of atrial epithelial cell.
C.C.	Central cavity.
C.C.F.	Central cavity forming.
C.P.	Calymmocyte cell-plug.
D.C. (Bas.)	Basophil region of daughter cell.
D.C.D. (A)	Degenerating daughter cell, separated from 'parent'.
D.C.D. (B)	Degenerating daughter cell, within parent blastomere.
D.C.F.	Daughter cell forming.
D.C.N.	Nucleus of daughter cell.
End. B.	Endodermal blastomere.
F.	Follicle.
F.C.	Follicular cavity.
F.E.	Follicular epithelium.
F.S.	Calymmocyte sheath.
I.C.	Incubation cavity.
I.F.	Incubation fold.
L.C.	Lateral cavity.
M.C.	Maternal blood-corpuscle.
N.B.	Neural blastomere.
N-C.C.	Neuro-chordal cell.
N-C.I.	Neuro-chordal invagination.
N-C.I.F.	Neuro-chordal invagination forming.
N.M.	Neural 'massif'.
Not. B.	Notochordal blastomere.
Not. M.	Notochordal 'massif'.
P.K.	Placental (embryonal) knob.
R.N.	Residual nucleus of blastomere.

develop within the 'unattached' region of the cell; the parent nucleus lies adjacent to the point of contact with the sheath. The process of division is initiated by the appearance within the cytoplasm of five or, as in the great majority of specimens, seven increasingly basophil sectors which fan out from the region of the parent nucleus. Each basophil region acquires a small nucleus which begins immediately to degenerate. The provenance of these nuclei is discussed below (see Discussion). Concurrently, the basophil massifs separate from the parent cell and the cytoplasm of each becomes increasingly positive to the Feulgen test.



Text-fig. 2. Section through the follicle at the eight-cell stage, before calymmocyte infiltration between the blastomeres has begun.



Text-fig. 3. Section through the follicle at the eight-cell stage, showing calymmocyte infiltration and endogenous budding.

After the regression of the nucleus is complete the DNA permeates the whole of the cytoplasm, then disappears. The nuclear regression and associated liberation of DNA may take place immediately before or after the daughter cells have become detached from the residual cell.

Throughout the whole of this process the residual cell region can be distinguished from the daughter cells by greater size, weaker basophily, and the possession of a larger nucleus. The residual cells can be readily distinguished from the calymmocytes, whether the latter form the follicular lining or are migratory, since all follicle cells, in contrast to the residual cells, at this stage are markedly smaller, have a relatively higher nuclear-cytoplasmic ratio, and possess a strongly basophil cytoplasm which contains numerous small, round mitochondria. The calymmocyte nuclei contain large amounts of DNA which is not in granular form, but dispersed in the nuclear sap. The transient daughter cells can also be easily distinguished from the calymmocytes in that the former have at all stages in their history an extremely low nuclear-cytoplasmic ratio, and during the greater part of their existence DNA is present in the cytoplasm, the amount increasing steadily as the cell ages. In the newly formed small nuclei the distribution of DNA is entirely normal and there is no evidence of the production of chromatic droplets indicative of cell degeneration (Wigglesworth, 1942). The significance of these observations in the light of the conflicting views of earlier authorities will be considered in the Discussion.

The daughter blastomeres ultimately degenerate completely into a mass of detritus and concurrently a normal but asynchronous division of the eight residual cells is initiated. Simultaneously the rate of calymmocyte proliferation increases and the spaces which earlier almost completely surrounded the blastomeres are invaded and obliterated by the follicle cells. Degeneration of the latter also begins. Thus, by now, the follicle presents a very complex picture. Its cavity has been reduced to a small slit and the blastomeres and the detritus resultant upon cell degeneration are embedded in a mass of normal calymmocytes.

The early blastophore to the period immediately subsequent to closure of the incubation fold (Text-fig. 10)

The blastophore to the early 'sixteen'-cell stage

As has been stated previously, the follicle or uterine sac lies adjacent to the atrial ectoderm. The continuing growth in size of the sac due to the increasing number of its cells causes it to thrust against, and subsequently to rupture, the atrial epithelium at one point. At the same time the sac changes from a round to a conical shape and projects into the cloaca as a small papilla. This structure is called by Brien (1928) the *cumulus proligère*; he reserves the term *blastophore* for a later stage in development when the incubation fold, about to be described, has closed over the papilla. This change in nomenclature seems to be rather unnecessary. Follicle cell proliferation continues far beyond the period of

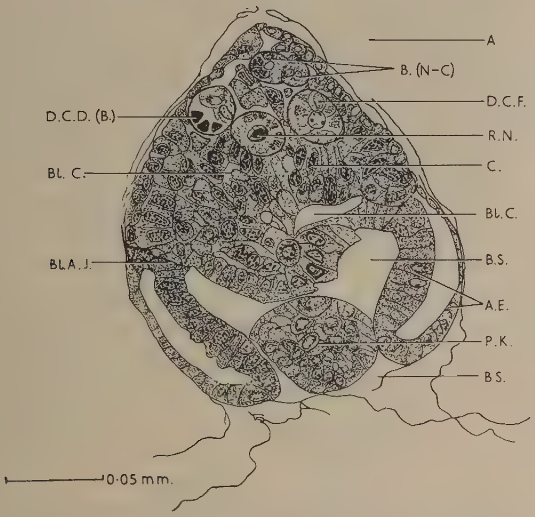
TABLE

The views of earlier authorities on certain features of the early embryonic development of Salpidae

	Todaro 1880	*Barrois 1881*	*Salensky 1883*	*Salensky 1917*	*Brooks 1893*	*Heider 1895*	*Metcalf 1897*	*Korotneff 1896, 1899*	*Brien 1928*	*Berrill 1950*
Blastomeres	Persist and form embryo and part of placenta	Persist and form embryo and part of placenta	Disappear	Persist and form part of embryo, major part of latter formed from follicle cells	Persist and form definitive embryo; primary embryo from follicle cells	Macromeres and micromeres form embryo and all placenta	. .	Unequal sizes. From four-cell stage many large blastomeres degenerate. Rest together with micromeres form embryo	Unequal sizes. Persist and form embryo	As Brooks
Intrablastomere subdivisions	Ingested yolky cells	Evidence of degeneration of blastomeres	Endogenous buds		Ingested follicle nuclei	Ingested follicle cells	Nuclei of follicle cells	Yolk deposits but *not* from ingested follicle cells	Associated with food passage from follicle cells to blastomeres	
Structure of early blastophore	. .	Follicle cells and blastomeres (see above)	Follicle cells	Follicle cells and blastomeres	Follicle cells and blastomeres (see above)	Blastomeres alone	. .	Follicle cells and blastomeres with regular arrangement	Follicle cells and blastomeres with regular arrangement	As Brooks
Functions of placental knob	Takes part in formation of blood-cells of embryo	Helps to nourish embryo	As Todaro		As Todaro	(*a*) Intermediary in passage of food from maternal blood to placenta (*b*) Second egg follicle	. .	As Todaro	No importance save to direct flow of blood to and from placenta	

upgrowth of the incubation fold; only late in development does it decline in intensity. Further, until a very late stage in development, the papilla contains blastomeres embedded among the follicle cells. In the present work the term *blastophore* is therefore used to describe the papilla from the time at which the follicle ruptures the atrial epithelium until the definitive embryo begins to take shape.

That the blastophore does not accommodate its increased girth in the relatively large maternal blood-sinus may seem to be somewhat surprising, since in this position the blastophore would be protected from the atrial water currents and at the same time be bathed in a nutrient fluid. It may be that the maternal blood-pressure is high and that it is thus easier for the follicle to thrust against the atrial epithelium than to expand into the sinus. Certainly, the more external position favours the birth of the oozooid. Within the cloaca it is doubly protected, since it lies within a double fold of the atrial wall and is further sheltered by a delicate double layered membrane, the incubation fold. This structure, as Barrois (1881) discovered, is of maternal origin. It bears a superficial resemblance to the vertebrate amnion and to the ectotrophe of *Hypsistozoa fasmeriana*, a compound ascidian, whose sexual development has been described by Brewin (1956). These latter membranes are, however, of embryonic origin.



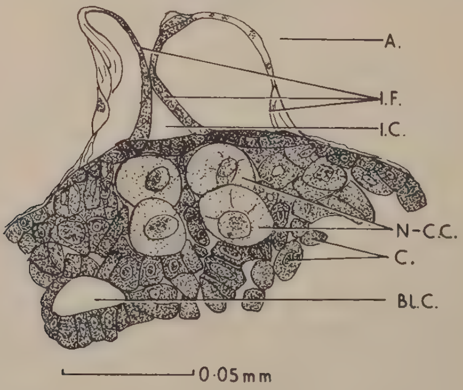
TEXT-FIG. 4. Vertical section through blastophore at 'sixteen'-cell stage to one side of junction of placental knob to the blastophore proper.

The incubation fold develops as follows. When the blastophore has completed its migration through the atrial epithelium the latter surrounds its base. In the intermediate zone between the cubical epithelium, which formerly over-laid the follicle, and the more laterally disposed pavement epithelium, a circular fold develops which grows up around the blastophore. Ultimately the fold whose circumference progressively decreases as it rises completely encloses the blastophore, but no apical fusion occurs. At 'birth', as the oozooid pushes its way out into the atrium, the intact fold slides down the sides of the animal and its attached placenta. It is difficult to ascribe a function to the incubation fold since a gymnogone salp, such as *T. democratica*, develops successfully although not completely enclosed in such a membrane.

During the period of upgrowth of the fold, development has continued within the blastophore. The original cavity has become occluded, and the number of blastomeres has increased to sixteen consequent upon the completion of the relatively normal division initiated after the termination of the phase of endogenous budding. The blastomeres become further separated from each other by migrating calymmocytes and there follows a second phase of budding. This, however, is less intense than the first and the daughter cells do not always separate from the parent cell before degeneration (Text-fig. 4). Ultimately sixteen scattered residual blastomeres remain.

The blastophore from the stage with sixteen residual blastomeres to the period immediately after the closure of the incubation fold

A large number of blastophores has been examined to determine whether the blastomeres are arranged in a definite pattern as Brien (1928) has suggested. Initially, there is no evidence of any plan. A little later, in all the blastophores of the same age, one group of cells can be observed to be occupying a constant position; the remainder are, as yet, disposed at random.



TEXT-FIG 5. Section through the apex of the blastophore, showing the neuro-chordal cells.

Immediately after the degeneration of the daughter cells resultant upon the second phase of budding, some of the residual blastomeres begin actively to migrate through small spaces left after calymmocyte degeneration. These blastomeres form a group of six or eight cells always located at the apex of the cone (Text-fig. 5). These are presumptive neural and notochordal cells. The presence of a transient notochord in the developing oozooid has not been noted by any of the earlier authorities. Its development, structure, and subsequent degeneration will be described in a later paper.

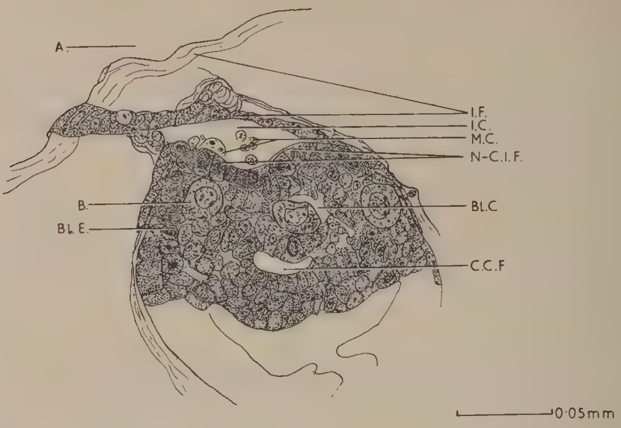
So far as the remaining blastomeres are concerned, the impression received from the study of a large number of blastophores at the 'sixteen'-cell stage is that the positions occupied by the blastomeres are largely determined by calymmocyte activity. Migrating calymmocytes thrust against the blastomeres, which are thus passively pushed into and through the small spaces left by other follicle cells which have degenerated.

By the time that the incubation fold is closing over the blastophore some of the blastomeres have divided or are in the process of division. These are cells which either by their own active movement, or by accident during the passive movement mediated by the calymmocytes, have come into contact with other blastomeres. It would appear that a contact stimulus is essential for the resumption of normal cleavage. This subject is considered further in the Discussion. The first to divide are, normally, the neuro-chordal cells, and thus a small neurochordal massif can be distinguished (Text-fig. 5). The innermost cells, i.e. those internal to the outer surface of the cone apex, are the notochordal cells.

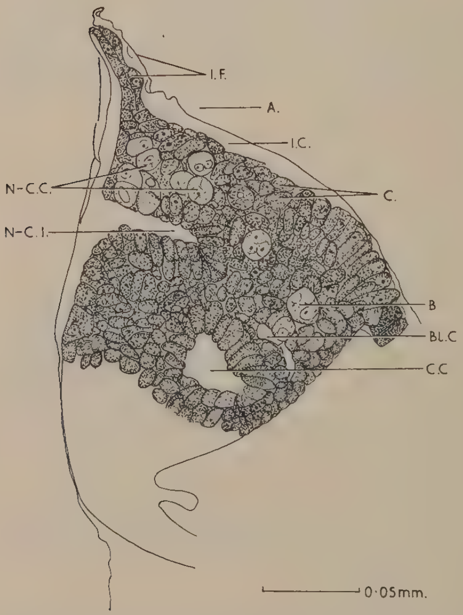
At this stage it is possible to discern the beginning of a pattern in the arrangement of the remaining blastomeres, which are increasing slowly in number and are now moving actively through the spaces provided by calymmocyte breakdown. The formation and positions of these spaces must therefore be described in order that the location of the blastomeres may be given with as much precision as possible.

Brien (1928) states: 'Au moment où les replis incubateurs se rejoignent dorsalement pour limiter la poche incubatrice, une cavité centrale apparaît dans le cumulus proligère en même temps que la fente représentant la cavité utérine virtuelle s'ouvre pour constituer une cavité supraplacentaire entre le plafond placentaire et la masse de l'embryon.' The central cavity 's'ouvre ultérieurement dans la cavité supraplacentaire'. With regard to the distribution of the blastomeres he says: 'A gauche et à droite, sous la cavité centrale se trouvent les blastomères pharyngiens, au-dessus de la cavité centrale dans le plan médian et latéralement, les blastomères ectoblastiques auxquels s'ajoutent antérieurement un massif médian, le massif nerveux et postérieurement un massif cloacal.' The observations made in the course of the present investigation are not entirely in accordance with those of Brien.

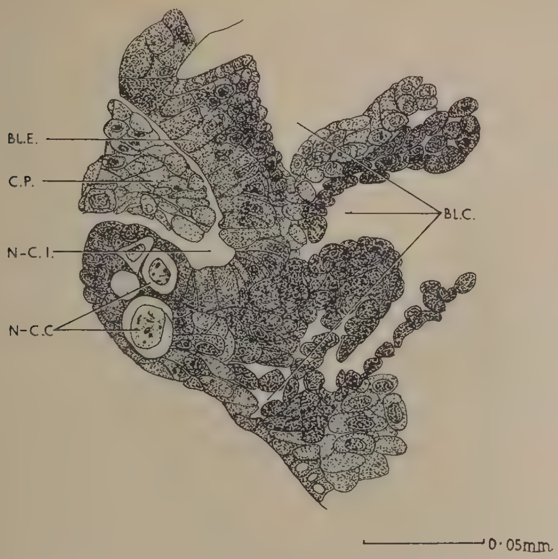
When the incubation fold has almost closed, a cavity appears somewhat subcentrally within the blastophore. Concurrently, at the cone apex, an invagination is initiated (Text-fig. 6), unobserved by Brien and the earlier authorities with the possible exception of Brooks (1893)—see p. 283. Apparently almost imme-



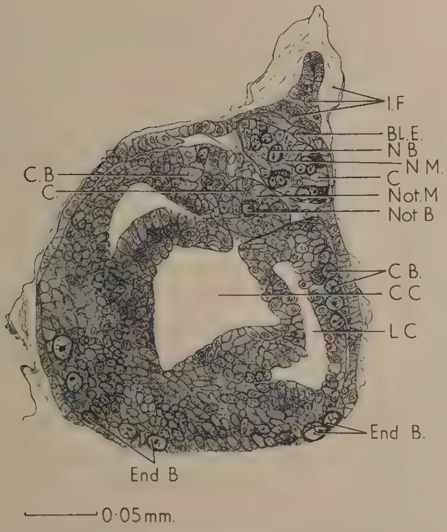
TEXT-FIG. 6. Vertical section through the apex of the blastophore, showing the beginning of the neuro-chordal invagination.



TEXT-FIG. 7. Vertical section, through anterior region of blastophore, showing late stage of neuro-chordal invagination, neuro-chordal cells on distal side of invagination.



TEXT-FIG. 8. Vertical section through region of neuro-chordal invagination, later than Text-fig. 7, neuro-chordal cells on proximal side of invagination.



TEXT-FIG. 9. Vertical section through the anterior end of the blastophore, showing the placental (blastophoral) cavities and neural and notochordal massifs (section slightly oblique).

diately this invagination, carrying the neuro-chordal cells with it, slides slightly downwards into what will be the future antero-dorsal region of the embryo (Text-figs. 7, 8). The details of the invagination are described below. The central cavity, which has the form of a tube with an extremely small lumen, extends in a direct line with, but ventrally to, the neuro-chordal cells in their original position. At the same time as the invagination slews to one side of the cone the incubation fold closes (in a few specimens the closure evidently occurred earlier), the central cavity increases in length, and the development of the lateral cavities begins (Text-fig. 9). All three arise as the result of calymmocyte degeneration. Almost immediately the central cavity acquires a cubical epithelium, the lateral spaces becoming similarly lined considerably later, i.e. when their basic development has been completed. At their inception, all three spaces are round in transverse section. In a few specimens the epithelium lining the central cavity extends so as apparently to form the lining of a closed tube between the central cavity and the maternal blood-sinus. In no specimen is this 'tube' patent, and indeed, with the almost immediate appearance of the *bouton placentaire* (see below) any open communication with the blood-sinus is precluded. All three cavities have as one of their functions the provision of routeways for blastomere migration. Transient and possibly accidental channels connect the central and lateral cavities; these are not to be confused with a permanent fusion which occurs much later. These impermanent channels are partially filled with the detritus produced by calymmocyte degeneration; at no time is there a definite epithelium and in some specimens only one channel appears to form, opening into either the right or left lateral space, whereas in others it seems highly improbable that any such channels develop. Later the lateral cavities fuse dorsally to form a single cavity with the shape of an inverted **U**, and part of this space will later be incorporated into the pharynx of the definitive oozooid. After the dorsal fusion has occurred the now tubular central cavity opens anteriorly and dorsally into the 'pharyngeal' space.

It is extremely difficult to equate the cavities noted by Brien with those which I have observed. It seems certain that Brien lacked material showing the stages of development between that at which the incubation fold has just closed and that in which the ground plan of the oozooid is discernible. He does not mention the lateral cavities in the text nor does he figure them. The space which he calls the central cavity throughout his text is, in fact, compounded of two separate spaces, the one homologous with the central cavity which I have observed, the other, only present in older specimens, lying above the pharynx and future gill-bar and, ultimately, forming part of the oozooid cloaca. In this I am therefore in accord with the interpretation of Heider (1895) and not with that of Brien.

The cavity said to be supraplacental by Brien will form, in part, the pharynx of the oozooid, and, in part, a cavity lying below it. The term 'supraplacental' does not seem to be suitable. The intimate apposition of maternal calymmocytes and embryonic blastomeres which constitutes the salp placenta occurs both dorsally and ventrally to the cavity. Indeed, the greater part of the placenta is dorsal. Therefore the term 'placental' cavity will be used to describe the space until the pharynx is formed from its dorsal sector.

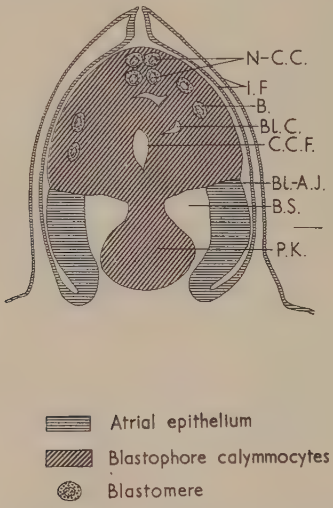
The fusion of the lateral cavities and the antero-dorsal channel between the central tube and the 'pharynx' occurs some time after the closure of the incubation fold. However, in order that a comparison between the cavities mentioned by Brien and those observed by me could be effected, reference had to be made to the later evolution of the spaces.

In the majority of the specimens examined when the incubation fold is closing or has just closed, from one to five blastomeres lie ventrally to the central cavity; in some specimens two groups are formed, in others all lie to one side. These are endoblasts which will ultimately, after many divisions, form the lining of the pharynx of the oozooid. Those situated dorsally, other than the neuro-chordal cells, are ectoblasts and are scattered at random. There is, at this stage, no mesoblastic spur; this structure does not develop, in fact, until the ground plan of the oozooid is clearly visible. The blastomere arrangement does not become precise until the lateral cavities have formed and are acquiring a definite epithelium.

There is, therefore, but slight agreement with Brien concerning the nature of the cavities and the distribution of the blastomeres within the placenta, but in an animal whose development is so complex a lack of material at critical stages can only too easily lead to the misinterpretation of observations, as I myself have found.

Two important phenomena occur immediately after or concurrently with the closure of the incubation fold. These are the development of the *bouton placentaire* (Brien, 1928) and the invagination affecting the neuro-chordal cells.

At the same time as the incubation fold closes there occurs a rapid proliferation of the follicle cells of the medio-ventral surface of the blastophore. As a result a 'knob' of cells projects into the maternal blood-sinus (Text-figs. 4, 10). Brien states that its function is to direct the flow of blood to and from the placenta. The cells comprising the *bouton* divide extremely actively and are strongly basophil. It seems therefore highly probable that they play a more active part in the economy of the developing placenta than Brien suggests. It is to be noted that while abundant supplies of glycogen are present in the maternal blood-cells within the sinus, no glycogen has been detected within the placenta. The placental knob stains deeply with neutral red and its cells contain numerous mitochondria, the number being considerably greater than that in the remaining follicle cells. It is perhaps also significant that the knob cells are strongly positive to Mancuso's test (1952) for glutathione, only very small amounts being present elsewhere within the placenta. It is suggested, tentatively, that the knob cells may be responsible for the elaboration of enzymes which convert at least some of the food brought to the blastophore by the maternal blood-corpuscles into a form suitable for the nutrition of both calymmocytes and blastomeres. It should be mentioned that since maternal corpuscles are present in very small numbers within the placenta itself and in the incubation cavity, the placental knob is not the sole means by which the food enters the blastophore. It is noteworthy that none of the corpuscles actually within the placenta contains glycogen; it may be that it is one of the functions of the knob, and of the outermost layer of cells elsewhere, to convert the glycogen into a simple sugar.



TEXT-FIG. 10. Diagrammatic longitudinal section through the blastophore, immediately prior to the closure of the incubation fold.

As has already been stated, the knob cells divide extremely actively, indeed their rate of division is higher than that of the blastomeres and of the remaining calymmocytes. The connexion between the knob and the blastophore proper is imprecisely demarcated, and careful examination shows that basophil cells, identical with those of the knob itself, extend into the placental base. There is, in fact, an apparently gradual change from the cells characteristic of the knob to the follicle cells typical of the blastophore. Further, the most dorsal knob cells are somewhat less basophil than those in its core. It therefore seems extremely probable that the knob forms a locus for calymmocyte proliferation, the cells so formed passing into the blastophore proper to replace those lost by degeneration, and becoming progressively less basophil during their inward passage. This activity of the knob suggests an alternative explanation for the large amounts of glutathione present. They may, possibly, be correlated with a high rate of respiratory metabolism, concomitant with the rapid increase in cell number and hence of protein synthesis. Such an explanation is to be preferred, since it is in the placental knob alone that the glutathione test is strongly positive, and while food is almost certainly passed into the placenta all over its surface, the very high rate of cell proliferation, as evidenced by the large numbers of mitotic figures to be seen in every section, is to be found only in the knob of the placenta.

The neuro-chordal invagination has the function of transferring the neuro-chordal cells to the correct position for their future development. The young blastophore in which sixteen residual cells are present is covered by a pavement or cubical epithelium composed of calymmocytes. Concurrently with the establishment of the group of neuro-chordal cells at the apex, the overlying epithelium becomes cubical, then columnar (Text-figs. 5, 6, 7), and almost immediately invagination begins (Text-figs. 5, 6). During this process the neuro-chordal cells are thrust to one or other side of the deepening fold. The invagination, as a result of the differential growth of the adjacent epithelium, slews over to one or other side of the cone apex (Text-fig. 7). The final position demarcates the future antero-dorsal region of the embryo. While it is not suggested that this invagination is in any way homologous with that occurring at the neurula stage of vertebrate embryogenesis, it is of interest to note that that of the salp is associated with underlying neuro-chordal cells.

For a short period after the invagination has closed there is a cavity within it. This is then occluded and, concurrently, a group of degenerating follicle cells is sloughed off from the outer surface of the blastophore in this region. The neuro-chordal cells will give rise to the cerebral ganglion, a nerve-cord above the gill-bar, and to the notochord; immediately after invagination they form a compact mass of cells internal to the placental epithelium and only later extend dorsally, along the length of the animal, consequent upon their apparently rapid proliferation.

It seems possible that this invagination is one of the 'foldings' observed by Brooks (1893): 'the Salpa embryo is blocked out in follicle cells which form layers and undergo foldings and other changes which result in an outline or model of all the general features in the organisation of the embryo'. He does not, however, specifically mention the neuro-chordal invagination in his text. It might perhaps be stated at this point that I do not accept his conception of a scaffolding of follicle cells around which the blastomeres organize themselves to form the definitive embryo. In some specimens the arrangement of the cavities which develop within the blastophore bears a superficial resemblance to that obtaining within the young oozooid, but whereas the former cavities are located within the body of the blastophore, the embryo develops distally and its body cavities incorporate only a part of some, and none of other, spaces within the blastophore. The walls of the early blastophore cavities are rather to be regarded as ladders up which the blastomeres climb to their definitive positions than as a scaffolding which, so to speak, preforms the oozooid.

The later development of *S. fusiformis* can be divided into two consecutive phases: (i) the migration of the blastomeres around and through spaces provided by calymmocyte degeneration into their definitive positions for the development of the oozooid; and (ii) the organogenesis and birth of the oozooid. These phases will be described in a later paper.

Some remarks upon the histology of the early blastophore

It may be advantageous to summarize the histology of the early blastophore since it has so far been described somewhat superficially.

Three types of cell can be distinguished clearly within the blastophore after staining with Heidenhain's iron haematoxylin and counterstaining with light green, and in preparations to which the Feulgen test has been applied.

The blastomeres. In blastophores containing more than sixteen blastomeres, these cells are larger than the calymmocytes, and their nuclear-cytoplasmic ratio is high. The large oval nucleus is surrounded by an extremely narrow rim of cytoplasm. Within the nucleus are small, irregularly shaped granules positive to the Feulgen test, and one or two large nucleoli. The nucleoplasm is reticulate and shows little affinity for any of the stains used. A small number of mitochondria is present. The division rate is low after the sixteen-residual-blastomere stage, but progressively increases as development proceeds.

The calymmocytes, other than those of the placental knob. Except in the young follicle or where the calymmocytes form epithelia, they are small oval cells with a nuclear-cytoplasmic ratio lower than that of the blastomeres. With age these cells become progressively less basophil; ultimately the majority become strongly acidophil. The nucleus is oval or round, small, and contains a few minute granules positive to the Feulgen test, and one nucleolus. Degeneration is either associated with swelling and vacuolation of the whole cell, or the cell may break down to form a 'mush'. The former process is characteristic of the later stages of development, but is not unknown in earlier stages.

The cells of the embryonal knob. These are small, strongly basophil cells which at all times have a very high rate of division, far in excess of that of the other follicle cells or of the blastomeres. Even in later stages, when the knob has attained its maximum size, the division rate is maintained. As has been stated previously, it seems probable that the products of the divisions of the knob cells pass into the blastophore proper to become, ultimately, the typical acidophil follicle cells there.

The nuclear-cytoplasmic ratio is high and the nucleus is normally round and resembles that of a blastomere. These cells can, however, be readily distinguished from blastomeres since the knob cells are always markedly smaller and, further, the cytoplasm contains a large number of small mitochondria.

DISCUSSION

The wider implications of salp reproduction in relation to chordate evolution will be discussed when the account of the development of *S. fusiformis* has been completed. However, three problems are raised by the study of the early sexual development of this species. They concern the two phases of endogenous budding, the functions of the placental or embryonic knob, and the nature and functions of the cavities appearing within the blastophore. The last two subjects have already been treated in some detail (see pp. 280–3). It therefore remains to discuss the significance of endogenous budding.

The Table shows that a number of theories have been proposed to explain the fragmentation of the early blastomeres. There are cogent reasons for accepting, in modified form, the view of Salensky (1917). It might also be noted that Todaro (1880) referred briefly to a process of blastomere gemmation.

No one previously has noticed that the 'fragments' are produced at two well-defined stages, i.e. at the eight- and at the 'sixteen'-cell stages. Korotneff (1899) states that from the four-cell stage it is difficult to count the number of blastomeres. This is not so, since the structure of the blastomeres is at all times markedly different from that of the follicle cells. There is little reason to doubt that Korotneff's 'micromeres' were, in fact, follicle cells, and it is therefore not surprising that he experienced difficulty in making a blastomere count. The other authorities do not mention later blastomere numbers. It appears to me highly improbable that only at the eight- and 'sixteen'-cell stages are follicle cells or their nuclei ingested (Todaro, 1880; Brooks, 1893; Heider, 1895; Metcalf, 1897), or yolk deposits formed (Korotneff, 1899). Immediately before and during the first period in which fragmentation occurs, the blastomeres, save at one point, are separated from the follicle cells by a space (Text-fig. 3). In no preparation have calymmocytes been observed to be crossing these spaces, or to be in the throes of ingestion at the one point of contact which, in any case, is at the opposite side of the cell from that at which the fragments are produced. Indeed, in all the specimens examined the calymmocyte sheath remains intact throughout the fragmentation process.

The number of portions developed by each blastomere is typically seven. It is extremely unlikely that such constancy of number is to be explained in terms of the ingestion of follicle cells by blastomeres or by the formation of yolk deposits; similarly, it would appear to be highly improbable that the newly ingested food should be voided so promptly and so uneconomically.

Each blastomere fragment contains one transient nucleus which, albeit small, is clearly to be distinguished from that of the 'parent' blastomere. Although divisions have been observed in the original blastomere nucleus during the early fragmentation stages, I cannot say with certainty that the daughter nuclei have been seen actually migrating into the cytoplasmic massifs just beginning to separate from each other although still attached at their bases to the 'parental' cytoplasm. This may be due to the intense basophily which accompanies the cytoplasmic division and to the small size of the nuclei themselves. I am, however, convinced that the daughter nuclei proliferated by the 'parent' are those subsequently to be found in the fragments. While the latter are degenerating, the 'parental' nucleus is completely inactive and there is but one nucleus in the residual region of the cell and no evidence of the degeneration of any of the proliferated nuclei at the place of origin.

The more recent statements of Brien (1928) concerning the nature of the fragments merit special consideration. His views are expressed in the following quotations: 'D'après nos préparations ces fragments protoplasmiques ont la même texture et la même colorabilité que le reste du protoplasme. S'ils sont parfois disposés selon les rayons d'un éventail, ils peuvent être beaucoup plus irréguliers . . . il s'agit d'une vacuolisation de la portion dilatée du blastomère. Contrairement aux lois de la segmentation certains blastomères tout au moins pendant les premiers stades, se dilatent dès qu'ils sont enveloppés par les cellules folliculeuses. . . .' This anomaly is 'due peut-être à une nutrition diffusive très intense. . . . Tous les blastomères ne se dilatent pas . . . les blastomères centraux restent homogènes'. It can be categorically stated that so far as *S. fusiformis* is concerned, *all* the blastomeres at the eight- and at the 'sixteen'-cell stages fragment. Resultant upon a slight asynchrony, some may begin to divide before others, and this may have caused Brien to assume that not all participate in the process. The fragments do not become vacuolated nor do they show the same *colorabilité* as the rest of the protoplasm. In contra-distinction to the cytoplasm of the rest of the cell, that of the fragments is intensely basophil and in later stages contains much DNA. Nuclei are to be observed and their presence cannot be explained in terms of 'une superposition des cellules situées en réalité dans des plans différents', which Brien suggests may have caused Salensky (1917), Brooks (1893), and Heider (1895) to have misinterpreted their preparations. The sections from which my observations were made were without exception cut at 2 μ and were particularly carefully examined; it can be stated unequivocally that nuclei are present in the fragments for a brief period, and their presence is not to be explained by a superposition of cells lying in different planes.

I have no doubt whatever that the fragments are daughter cells produced by a process of endogenous budding. It appears to me that there are two possible explanations of this rather remarkable phenomenon.

The separation of the blastomeres by migrating follicle cells may inhibit normal division by virtue of an inhibitory substance produced by the calymmocytes or because contact stimuli received from adjacent blastomeres are essential for regular cleavage. This latter view receives support from the observation that, in later development, blastomere divisions are not resumed until, as a result of active or passive migration, the blastomeres come into contact with each other.

A second possible explanation is that salps exhibit polyembryony, i.e. each parent blastomere at the eight- and later at the 'sixteen'-cell stage is capable of developing into an oozooid. Such a theory would postulate the secretion of a substance, presumably by the follicle cells, which inhibits the production of a multiplicity of embryos associated with a single placenta; and that the blastomeres, each finding itself unable to develop into an oozooid, instead combine to form a single offspring. The second but feebler phase of endogenous budding would, presumably, be a second attempt to produce a number of oozooids, the first having failed. Inherent in this theory is the acceptance of the idea that all the early blastomeres, i.e. to the 'sixteen'-cell stage, are potentially able to give rise to embryos. It is of interest in this connexion that in some species of salp more than one egg may be present in the ovary, e.g. four in *S. hexagona* (Brooks, 1893) and 4–6 in *S. zonaria* (Korotneff, 1896*a*). In addition, I have found in one specimen of *S. fusiformis* two embryos attached to the same placenta; both were almost fully developed. It might be suggested that in the course of their evolution the salps have progressively lost the capacity to produce normally more than a single embryo at one time, but that a vestige of this ability persists in the phenomenon of blastomere budding.

It is true that the Ascidians, from which I am convinced that salps have evolved (my reasons will be given in a subsequent paper), normally produce large numbers of offspring. However, in viviparous forms the number is much reduced and in *H. fasmeriana*, whose development has been described by Brewin (1956), only one is produced at a time. The embryo begins its development within an ovarian follicle which has previously migrated into the oviduct, and whose walls proliferate follicle cells. These, however, while serving to nourish the embryo, do not separate the blastomeres. It would thus appear that the reduction in the number of embryos produced by a single parent is not necessarily associated with the suppression of the development of all but one of a number of embryos by invading follicle cells which secrete an inhibitory substance. Moreover, the condition in *S. hexagona* and *S. zonaria* is very different from polyembryony. The several ova are present in the ovary before fertilization and are not produced *ab initio* from a single fertilized ovum in the already migrated follicle.

I therefore incline to the former of the two hypotheses and see the origin of endogenous budding in salps as an extension of the calymmocyte proliferation observed in *H. fasmeriana* and doubtless present in many other ascidians not yet investigated. The process, which might almost be said to have 'run wild' in the salps, results in blastomere separation. I am of the opinion that it is the resultant lack of contact stimuli, rather than the action of an inhibitory substance secreted by the calymmocytes, which is responsible for the aberrant divisions which take the form of endogenous budding. Nevertheless, this view is far from being final. In the near future I hope to perform experiments upon suitable material of *S. fusiformis*, material from which blastomeres will be isolated by chemical means. It is hoped thereby to discover whether in such conditions endogenous budding or normal divisions occur, and also whether there is any suggestion of the development of embryos from any of the isolated cells.

SUMMARY

The early sexual development of *S. fusiformis* Cuvier is described.

The fertilized egg begins to develop in the single ovarian follicle, dorso-laterally, close to the atrial epithelium. At the eight-cell stage the follicle cells (calymmocytes) proliferate rapidly and insinuate themselves between the blastomeres. Each blastomere at this point buds endogenously and the resultant seven daughter cells rapidly degenerate; a large residual cell persists. The calymmocytes continue to increase in number but their degeneration also is initiated. The follicle perforates the atrial epithelium, and is now known as the blastophore. A normal blastomere division occurs and is followed by a second phase of budding and daughter-cell degeneration. A structure, the placental knob, develops ventrally; one of its functions is to proliferate calymmocytes. The blastomeres begin to move around regularly and irregularly occurring spaces produced by calymmocyte degeneration, and, on gaining contact with each other, normal division is resumed. A medio-dorsal, neuro-chordal cell massif becomes recognizable and subsequently it moves antero-dorsally. Three regularly occurring cavities begin to develop during the neuro-chordal cell movement. They are centrally and laterally placed and will ultimately fuse and form, in part, the oozooid pharynx. The remaining blastomeres aggregate in groups which will give rise to the cloacal epithelium, remaining ectoderm, and endoderm. Mesodermal cells are not yet to be discerned with certainty. During the early stages of development of the three cavities an incubation fold develops from the adjacent atrial epithelium. It grows up around the blastophore. The aggregation of the cloacal, ectodermal, and endodermal blastomeres and the fusion of the central and lateral cavities occur after the closure of the fold.

The significance of certain phenomena, in particular of endogenous budding, are discussed. The conclusion favoured at present is that the budding process is an abnormal phase of division induced by a loss of blastomere contact.

RÉSUMÉ

Le développement sexuel de Salpa fusiformis *Cuvier. 1ère partie*

Ce travail décrit le début du développement sexuel chez *Salpa fusiformis*. L'œuf fécondé commence à se développer dans l'unique follicule ovarien en position dorso-latérale près de l'épithélium atrial. Au stade à huit cellules les cellules folliculeuses (calymmocytes) prolifèrent rapidement et s'insinuent entre les blastomères. A ce moment, chaque blastomère est le siège d'un bourgeonnement endogène et les sept cellules filles qui en résultent dégénèrent rapidement; une grande cellule résiduelle persiste. Les calymmocytes continuent à augmenter en nombre mais se mettent également à dégénérer. Le follicule perfore l'épithélium atrial et est alors connu sous le nom de blastophore. Une division normale des blastomères se produit et est suivie par une seconde phase de bourgeonnement avec dégénérescence des cellules-filles. Une nouvelle structure, le bouton placentaire, se développe ventralement; une de ses fonctions est de produire par prolifération des calymmocytes. Les blastomères commencent à se déplacer autour d'espaces tantôt réguliers, tantôt irréguliers que produit la dégénérescence des calymmocytes et lorsqu'ils reprennent contact avec d'autres, les divisions normales réapparaissent. Un massif cellulaire médio-dorsal, à destinée neuro-chordale, devient reconnaissable et se déplace ensuite vers l'avant et dorsalement. Trois cavités régulièrement observables commencent à se développer pendant le mouvement des cellules neuro-chordales. Elles occupent la position centrale et les deux régions latérales et fusionnent finalement pour former une partie du pharynx de l'oozoïde. Les blastomères qui ont subsisté s'agrègent en groupes qui donnent naissance à l'épithélium cloacal, au reste de l'ectoderme et à l'endoderme. On ne peut encore reconnaître avec certitude des cellules mésodermiques. Pendant que les trois cavités commencent à se développer un repli incubateur se constitue aux dépens de l'épithélium adjacent de l'atrium et s'accroît autour du blastophore. L'agrégation des blastomères formateurs du cloaque, de l'ectoderme et de l'endoderme et la fusion des cavités centrale et latérales sont des transformations postérieures à la fermeture du repli. La signification de certains processus est discutée, en particulier celle du bourgeonnement endogène. La conclusion envisagée actuellement avec le plus de faveur est que le phénomène de bourgeonnement est une phase de division anormale induite par la perte du contact entre les blastomères.

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L'Inhibition spécifique de la différenciation du cerveau des Planaires d'eau douce en régénération

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AVEC UNE PLANCHE

LA régénération des Planaires d'eau douce est caractérisée par la formation d'un blastème dû à l'accumulation de néoblastes. Ces cellules, à caractère embryonnaire, migrent vers la blessure puis se différencient pour reconstituer les tissus excisés (Wolff & Dubois, 1947; Dubois, 1949). Les tissus de la Planaire peuvent intervenir dans les processus de différenciation en favorisant l'apparition d'un organe. Ainsi le cerveau induit la régénération des yeux de la Planaire *Polycelis nigra* par l'intermédiaire de substances chimiques diffusibles (Wolff & Lender, 1950; Lender, 1952, 1956*a*). Mais d'autre part certains auteurs ont mis en évidence des phénomènes d'inhibition dans la régénération des Planaires. Morgan (1902) et Rand & Ellis (1926) constatent qu'une tête inhibe la régénération d'une autre tête chez les Planaires bicéphales. Rand & Browne (1926) et Miller (1938) empêchent la régénération de la tête en greffant une région céphalique dans la région antérieure de la Planaire opérée. Chevtchenko (1937) constate que la tête de l'hôte inhibe le développement d'une région céphalique dans un greffon de tête implanté dans la région antérieure de la Planaire. L'inhibition ne se manifeste pas si le greffon est implanté dans la région caudale. Il semble donc que la tête des Planaires d'eau douce soit capable, dans certaines conditions, d'inhiber la différenciation d'une autre tête.

Brøndsted (1946, 1956) a montré que chez certaines Planaires d'eau douce il existe un 'time-graded regeneration field'. Dans ce champ les différents points ont des pouvoirs de régénération différents. Au moment de la régénération le point à pouvoir de régénération le plus élevé se différencie d'abord et inhibe la formation de structures similaires.

D'autre part, si on élève des Planaires en présence de broyats de têtes, on constate que la régénération du cerveau est inhibée par ces broyats. Par contre, en présence de broyats de queues, la régénération des ganglions cérébroïdes se déroule normalement (Lender, 1955, 1956*b*, 1958).

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J'ai poursuivi l'analyse de ces phénomènes, afin de mettre en évidence le tissu inhibiteur ainsi que la manière dont il intervient.

MÉTHODES ET TECHNIQUES

Deux espèces de Planaires d'eau douce ont été soumises à l'expérimentation: *Dugesia (Euplanaria) lugubris* (O. Schm.) et *P. nigra* (Ehrb.). Ces espèces sont élevées régulièrement au laboratoire.

La température de l'eau d'élevage des Planaires opérées était de 18° C.

Les expériences peuvent être groupées en deux séries: les expériences de greffes et les expériences sur l'action des broyats.

Expériences de greffes

Les Planaires *D. lugubris* sont opérées sous anesthésie au chlorétone à 2 pour mille. Un cerveau est prélevé sur un individu et greffé sur un hôte à la hauteur des ovaires, juste en arrière du cerveau. Un à deux jours plus tard, le cerveau de l'hôte est excisé ou la Planaire est décapitée en avant de la greffe. Le pouvoir de régénération est contrôlé par l'observation de la régénération des yeux.

Expériences avec les broyats

On prélève la région céphalique de la Planaire en sectionnant la tête à la hauteur des ovaires. Une section à la hauteur de l'orifice génital permet de prélever la région caudale. Les fragments prélevés sont broyés dans un microbroyeur. On obtient le broyat brut à raison de 20 têtes par 10 c.c. d'eau. Actuellement je lyophylise ces broyats et je conserve la poudre à la température de —20° C. Ce procédé n'altère pas l'activité de la substance.

Le broyat en suspension dans l'eau peut être centrifugé à 10.000 g. pendant 30 minutes. Le surnageant est limpide et légèrement visqueux. Le culot est dense; il contient les pigments et les débris cellulaires du broyat.

Les Planaires *D. lugubris* ou *P. nigra*, après décapitation ou excision du cerveau, sont élevées par 4 dans 10 c.c. de broyat. Le broyat est renouvelé tous les deux ou trois jours pendant 9 jours. En même temps le blastème de régénération est légèrement incisé pour favoriser la pénétration des substances actives.

L'élevage de *D. lugubris* a été fait en présence de broyats d'animaux de la même espèce. Pour *P. nigra* j'ai utilisé soit des broyats de têtes d'animaux de la même espèce, soit des broyats de têtes de *D. lugubris*, sans constater de différences dans les résultats de l'expérience.

Les animaux témoins sont opérés de la même manière mais on les élève par 4 dans 10 c.c. d'eau normale en l'absence de broyat. Après 9 jours, les Planaires sont fixées au Zenker, incluses dans la paraffine et coupées à 5 μ. Les coupes sont colorées au Mallory.

RÉSULTATS

La régénération de la tête en présence d'un greffon de cerveau

Vingt greffes de cerveau ont été implantées dans la région antérieure de *D. lugubris*. Après décapitation de la Planaire hôte, le greffon peut évoluer de deux manières différentes.

Dans 10 expériences le cerveau greffé devient la tête de la Planaire. Macroscopiquement on ne peut plus distinguer les tissus de l'hôte de ceux du greffon. La forme de la tête est normale et la Planaire a régénéré les deux yeux. Sur les coupes histologiques on distingue le cerveau greffé. Il est en continuité avec les troncs nerveux de l'hôte. Ce cerveau est long en moyenne de 337 μ. Les longueurs extrêmes observées sont 150 μ et 500 μ. La régénération du cerveau de l'hôte est inhibée.

TABLEAU 1

Régénération de la tête de D. lugubris *après implantation d'un greffon de cerveau (en μ)*

Longueur du cerveau greffé	*Longueur de la tête régénérée*	*Longueur du cerveau régénéré*	*Longueur du cerveau régénéré chez les témoins*
260	150	75	
275	265	100	
300	330	125	
380	100	0	
300	100	0	250 à 400 (16 planaires)
350	200	0	
360	450	50	
170	450	0	
300	200	75	
240	130	60	
Moyenne 294	238	49	326

Dans 10 autres expériences (Tableau 1) la tête de l'hôte se régénère. Le greffon, contenant le cerveau, se développe aussi et reste attaché à la face ventrale ou dorsale de l'hôte par sa région postérieure. A la fin de l'expérience le greffon a différencié 2 yeux normaux et 1 à 3 yeux supplémentaires. Son cerveau est développé normalement, la longueur moyenne des ganglions cérébroïdes est de 294 μ; les chiffres extrêmes sont 170 et 380 μ.

Le régénérat céphalique de l'hôte a différencié deux yeux normaux et 1 à 3 yeux supplémentaires. La longueur de la tête régénérée varie entre 100 et 450 μ. La longueur moyenne se situe aux environs de 238 μ. Cette tête présente dans le parenchyme une accumulation de néoblastes. Dans 4 expériences aucune cellule nerveuse ne s'est différenciée. Les troncs nerveux sectionnés se prolongent par des massifs de néoblastes indifférenciés. Dans les 6 autres expériences on distingue du tissu nerveux sur une longueur de 50 à 125 μ. Donc en moyenne le tissu nerveux s'est différencié sur une longueur de 49 μ. L'inhibition de la régénération du cerveau apparaît très nettement si on fait la comparaison avec le cerveau régénéré des Planaires témoins. En 9 jours les individus témoins ont régénéré un cerveau long de 250 à 400 μ, en moyenne 326 μ.

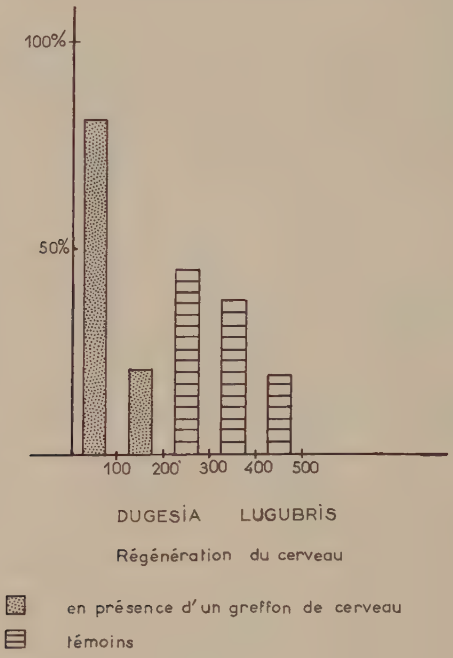

FIG. 1

Nous constatons donc (Fig. 1), qu'en présence d'un greffon de cerveau, la Planaire *D. lugubris* décapitée peut régénérer une tête. La régénération des yeux est induite par le cerveau du greffon. Mais cette tête n'a pas régénéré de cerveau de longueur normale. Si on obtient un début de différenciation, ce cerveau est beaucoup plus court que celui des témoins. Le cerveau greffé inhibe la régénération du cerveau de l'hôte. La tête se développe mais la différenciation des cellules nerveuses est inhibée.

Dans ces expériences le greffon de cerveau est très proche du blastème de régénération de l'hôte. On pourrait penser qu'une compétition s'établit entre les deux. Dans ce cas l'inhibition serait due à un déficit de matériel de régénération et le cerveau de l'hôte ne pourrait pas se différencier. Mais les expériences avec les broyats vont montrer qu'un autre mécanisme doit être mis en cause pour expliquer l'inhibition de la régénération du cerveau.

La régénération de la tête en présence de broyats

Les expériences sur la régénération des yeux de *P. nigra* ont montré que le cerveau est l'inducteur de leur différenciation grâce à l'émission de substances chimiques spécifiques (Lender, 1956*a*). Des expériences antérieures (Lender, 1955, 1956*b*) avaient montré que le pouvoir inhibiteur était aussi lié à la diffusion de substances chimiques émises par le cerveau. J'ai complété ces recherches en étudiant plus spécialement l'activité des broyats centrifugés. Les expériences ont été faites sur *D. lugubris* et *P. nigra*. Ces Planaires, après ablation du cerveau, sont élevées dans différents types de broyats: broyats bruts de têtes et de queues, broyats centrifugés de têtes et de queues. Pour *Polycelis* j'ai utilisé soit des broyats de têtes d'animaux de la même espèce, soit des broyats de têtes de *D. lugubris*.

Expériences sur D. lugubris

Les résultats numériques sont rapportés dans le Tableau 2.

Si on élève *D. lugubris* en présence de broyats bruts de têtes, on observe une inhibition de la régénération du cerveau excisé. Cinq Planaires sur 30 n'ont pas régénéré de cerveau. La majorité des autres individus ne différencie qu'une faible longueur de tissu nerveux, 97 μ en moyenne, c'est-à-dire 3 fois moins que la longueur moyenne du cerveau régénéré par les Planaires témoins (319 μ), élevées en l'absence de broyats. L'inhibition de la régénération du cerveau est due à une action spécifique, car, si on utilise un broyat brut de queues, le cerveau se différencie normalement dans la Planaire opérée. Sa longueur moyenne est de 309 μ.

TABLEAU 2

Régénération du cerveau de D. lugubris

		Planaires en expérience		*Planaires ayant régénéré des yeux*	*Absence de régénération de cerveau*	*Régénération du cerveau*		
		Début	*Fin*			*Nombre*	*Longueur (en μ)*	*Moyenne (en μ)*
Broyat de têtes	brut	32	30	30	5	25	45 à 375	97
	centrifugé surnageant	44	41	41	7	34	50 à 230	120
	centrifugé culot	16	16	16	0	16	200 à 400	302
Broyat de queues	brut	24	15	15	0	15	150 à 450	309
	surnageant de la centrifugation	24	24	24	0	24	230 à 500	332
Témoins sans broyats		80	56	56	0	56	150 à 500	319

Ces expériences montrent donc clairement que l'activité inhibitrice du cerveau est indépendante de son intégrité anatomique. Elle doit s'exercer par l'intermédiaire de substances diffusées dans l'eau d'élevage. Pour éliminer au maximum les débris cellulaires, on peut centrifuger les broyats de têtes à 10.000 g. pendant 30 minutes. On éprouve les deux fractions obtenues, le surnageant et le culot, en y élevant des Planaires sans cerveau. Le surnageant contient la substance inhibitrice. Sept *D. lugubris* sur 41 ne régénèrent pas de tissu nerveux. Les 34 autres individus ont régénéré du tissu nerveux sur une longueur de 120 μ en moyenne. Ce chiffre est comparable à celui qu'on obtient dans les expériences avec les broyats bruts. Le culot de la centrifugation des broyats de têtes ne manifeste aucune activité inhibitrice. Toutes les Planaires opérées régénèrent un cerveau long en moyenne de 302 μ (319 μ pour les Planaires témoins).

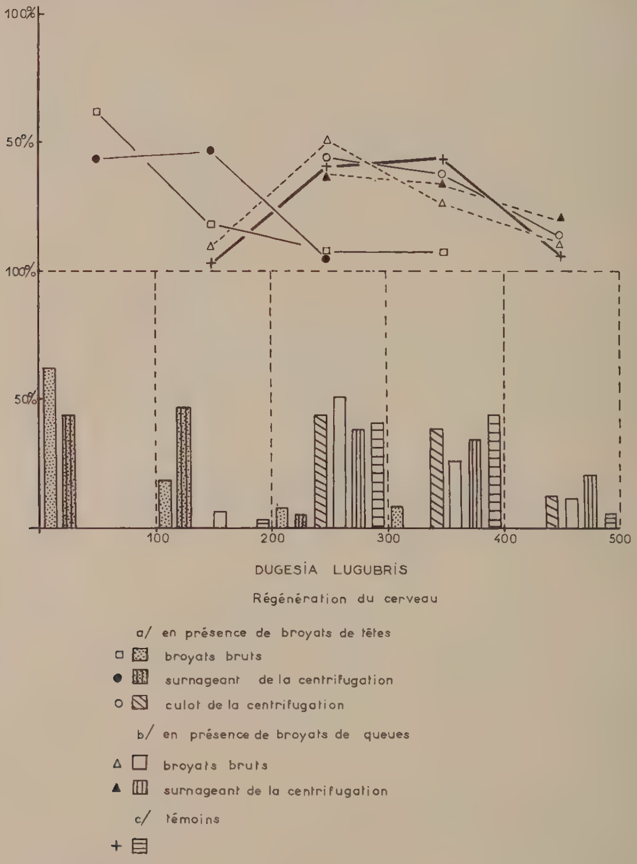

FIG. 2

Les expériences avec le surnageant des broyats de queues confirment les résultats qu'on obtient avec les broyats bruts de queues. Ce surnageant est inactif. Le cerveau se régénère. Il est long en moyenne de 332 μ.

La Fig. 2 résume l'ensemble des résultats obtenus chez *D. lugubris*. Le broyat brut de têtes et le surnageant des broyats de têtes manifestent une activité inhibitrice de la régénération du cerveau. Les broyats de queues et le culot de la centrifugation des broyats de têtes sont inactifs. La régénération du cerveau de *D. lugubris* est donc inhibée par des substances chimiques, émises par le cerveau. Ces substances se concentrent dans le surnageant de la centrifugation des broyats de têtes.

Ces résultats sont confirmés par les expériences sur *P. nigra.*

Expériences sur P. nigra

Les expériences précédemment décrites ont été refaites sur *P. nigra* (Tableau 3).

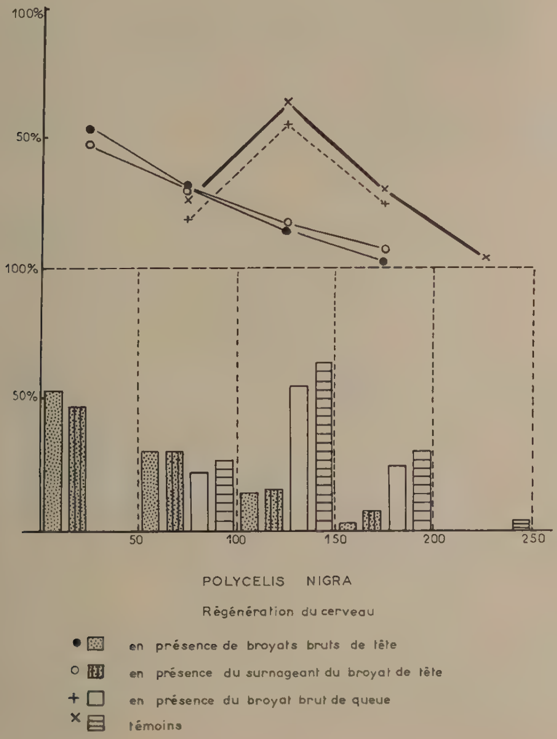

FIG. 3

Les broyats bruts de têtes de *P. nigra* ou *D. lugubris* inhibent la régénération du cerveau de *P. nigra.* Quinze Planaires sur 43 ne différencient pas de cerveau (Pl., figs. A, B). Les autres régénèrent du tissu nerveux sur une longueur moyenne de 60 μ. Pendant le même temps les *Polycelis* élevées en l'absence de broyats régénèrent un cerveau long en moyenne de 130 μ (Pl., fig. C). Le surnageant de la centrifugation des broyats de têtes inhibe aussi la différenciation du cerveau. Treize individus sur 40 ne régénèrent pas de cerveau; les autres différencient un tissu nerveux long de 57 μ en moyenne (Pl., fig. D). Les broyats bruts de queues ne manifestent pas d'activité inhibitrice de la régénération du cerveau. Dans ce cas le cerveau régénéré est long en moyenne de 115 μ. La Fig. 3 met en évidence le pouvoir inhibiteur des broyats bruts de têtes et du surnageant de la centrifugation des broyats de têtes.

TABLEAU 3

Régénération du cerveau de P. nigra

		Planaires en expérience		*Planaires ayant régénéré des yeux*	*Absence de régénération de cerveau*	*Régénération du cerveau*		
		Début	*Fin*			*Nombre*	*Longueur (en μ)*	*Moyenne (en μ)*
Broyat de têtes	brut	52	43	43	15	28	25 à 160	60
	surnageant de la centrifugation	48	40	40	13	27	35 à 150	57
Broyat de queues		12	9	9	0	9	70 à 160	115
Témoins sans broyats		52	43	43	0	43	60 à 205	130

DISCUSSION

Les expériences décrites montrent que, chez les Planaires d'eau douce, *D. lugubris* et *P. nigra*, le cerveau inhibe la différenciation d'une autre masse cérébroïde. Une Planaire décapitée régénère une tête oculée. Mais en présence d'un greffon de tissu nerveux, le cerveau de l'hôte ne se différencie pas ou se différencie imparfaitement. Les cellules de régénération reconstituent les tissus de l'hôte sauf le cerveau. La place de celui-ci est occupée par des néoblastes dont la différenciation a été inhibée. L'activité inhibitrice est organo-spécifique, car les tissus non nerveux se différencient normalement.

D'autre part, l'activité inhibitrice est liée à la présence de substances chimiques émises par le cerveau de la Planaire. Ces substances sont présentes dans le broyat de têtes de *D. lugubris* et de *P. nigra*. La différenciation du cerveau de *P. nigra* est inhibée d'une manière identique par des broyats de têtes de *P. nigra* ou de *D. lugubris*. Les substances inhibitrices ne manifestent donc pas de spécificité zoologique.

Les substances inhibitrices de la différenciation du cerveau diffusent dans l'eau et sont contenues dans le surnageant de la centrifugation du broyat. Le culot, contenant le pigment et les débris cellulaires, est inactif. La région caudale ne contient pas les substances inhibitrices. La diffusion de ces substances dans le corps de la Planaire doit donner naissance à un gradient d'inhibition antéro-postérieur. Child et ses élèves ont déjà mis en évidence un gradient morphogénétique chez les Planaires. Mais pour cette école le gradient morphogénétique se confondait avec un gradient métabolique. Or mes expériences montrent pour la première fois que le gradient morphogénétique des Planaires dépend de la diffusion de substances inhibitrices émises par le cerveau. Dans la région céphalique le cerveau inhibe la différenciation d'une autre masse cérébroïde. Au fur et à mesure qu'on s'éloigne de la région antérieure l'activité inhibitrice du cerveau s'atténue. Dans la régon caudale elle ne se manifeste plus; les substances inhibitrices ne diffusent pas jusque dans la queue.

Ces résultats sont aussi en accord avec ceux obtenus par Brøndsted (1946, 1956). Cet auteur a en effet montré que les Planaires d'eau douce possédaient un 'time-graded regeneration field'. Dans ce champ, le point, présentant la différenciation la plus poussée, exerce une inhibition qui empêche la régénération d'une structure analogue. D'après mes expériences, le cerveau jouerait ce rôle et empêcherait la différenciation d'un autre cerveau grâce à la diffusion de substances chimiques inhibitrices.

Les expériences de greffes de Chevtchenko (1937) et Lender (1950) corroborent l'existence de ce gradient d'inhibition. Un greffon, implanté à différents niveaux du corps d'une Planaire, différencie d'autant plus facilement un cerveau que la greffe est plus éloignée de la tête de l'hôte.

L'inhibition spécifique d'un organe par un tissu homologue a aussi été mise en évidence dans d'autres groupes d'animaux. Tardent (1955, 1956), après Rose & Rose (1941) et Steinberg (1954), a montré que la régénération des hydranthes de *Tubularia* pouvait être inhibée par des substances émises par un autre hydranthe. Tardent & Eymann (1958) ont recherché les propriétés de la substance inhibitrice produite par *Tubularia*. Elle est thermostable, soluble dans l'eau et le méthanol, insoluble dans l'acétone et l'éther.

Rose (1955) a aussi mis en évidence des inhibitions spécifiques dans le développement embryonnaire des organes de *Rana pipiens*. Le cerveau inhibe le développement du système nerveux, le cœur produit des anomalies dans le développement du cœur par exemple.

Il semble donc que l'inhibition organo-spécifique soit un phénomène assez répandu, pouvant intervenir dans le développement embryonnaire et la régénération.

Le rôle morphogénétique de la substance inhibitrice de la différenciation du cerveau pendant la régénération normale des Planaires pourrait se concevoir de la manière suivante:

Après décapitation de la Planaire, le bourgeon de régénération s'édifie et différencie un cerveau aux dépens des néoblastes. Ce cerveau, régénéré en premier lieu, émet des substances inhibitrices qui empêchent la différenciation d'un deuxième cerveau.

Le cerveau joue donc un rôle prépondérant dans la différenciation d'un régénérat de Planaire d'eau douce. Il inhibe la différenciation de toute autre masse cérébroïde et il induit la différenciation des yeux (Lender, 1956*c*).

RÉSUMÉ

1. Pendant la régénération de la tête des Planaires d'eau douce, le cerveau de *D. lugubris* et de *P. nigra* inhibe la différenciation d'une autre masse cérébroïde.

2. En présence d'un greffon de cerveau, la Planaire *D. lugubris*, décapitée, régénère une tête. La régénération du cerveau est inhibée totalement ou partiellement.

3. Le pouvoir inhibiteur du cerveau est dû à la sécrétion de substances chimiques par cet organe. Ces substances peuvent être mises en évidence dans les broyats bruts de têtes et dans le surnageant de la centrifugation des broyats de têtes. Les broyats de queues sont inactifs.

4. Les substances inhibitrices ne présentent pas de spécificité zoologique. Un broyat de têtes de *D. lugubris* inhibe la différenciation du cerveau de *P. nigra*.

5. Les substances inhibitrices sont organo-spécifiques. En présence d'un greffon de cerveau ou d'un broyat de têtes, la Planaire décapitée régénère les tissus de la tête sauf le cerveau.

6. Le gradient antéro-postérieur de diffusion des inhibiteurs organo-spécifiques du cerveau correspond au gradient morphogénétique de Child.

SUMMARY

1. During regeneration of the head of the fresh-water Planarians *Dugesia lugubris* and *Polycelis nigra* a brain inhibits the differentiation of another brain.

2. When bearing a graft of a brain, a decapitated *D. lugubris* regenerates a head, but the regeneration of the brain is totally or partly inhibited.

3. The inhibitory power of the brain is due to its secretion of chemical substances. These can be demonstrated in crude homogenates of heads and in the supernatant of centrifuged homogenates of heads. Homogenates of tails are inactive.

4. The inhibitory substances are not species-specific. A homogenate of *D. lugubris* heads inhibits brain differentiation in *P. nigra*.

5. The inhibitory substances are organ-specific. In the presence of either a brain graft or a homogenate of heads, a decapitated Planarian regenerates all the head tissues except the brain.

6. The antero-posterior diffusion gradient of organ-specific brain inhibitors corresponds to the morphogenetic gradient of Child.

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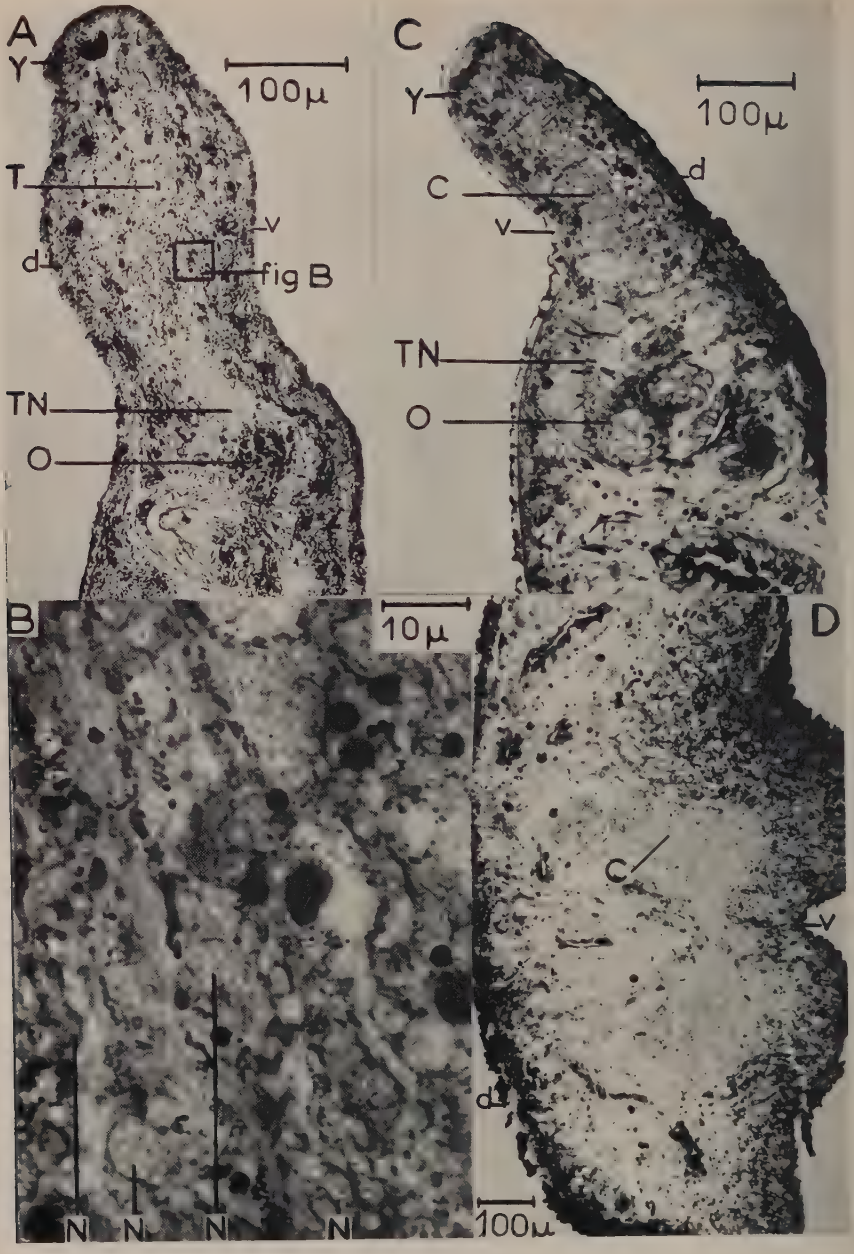

TH. LENDER

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EXPLICATION DE LA PLANCHE

FIG. A. Photographie d'une coupe sagittale de *P. nigra.* Après excision du cerveau, la Planaire a été élevée pendant 9 jours en présence de broyats bruts de têtes. Le cerveau ne s'est pas régénéré; le tronc nerveux latéral (*TN*) ne pénètre pas dans le tissu régénéré (*T*).

FIG. B. Agrandissement du tissu régénéré encadré dans la fig. A. A la place du cerveau on distingue de nombreux néoblastes (*N*) non différenciés.

FIG. C. Photographie d'une coupe sagittale de *P. nigra.* Planaire témoin, élevée après excision du cerveau en l'absence de broyats pendant 9 jours. On distingue le cerveau régénéré (*C*) en prolongement du tronc nerveux latéral (*TN*).

FIG. D. Photographie d'une coupe transversale de *P. nigra.* La Planaire, après décapitation, a été élevée en présence du surnageant de la centrifugation d'un broyat de têtes. Le cerveau régénéré (*C*) est long de 25 μ et beaucoup moins large que le cerveau normal.

Légende commune aux quatre figures: *d*, épiderme dorsal; *o*, ovaire; *v*, épiderme ventral; *y*, œil.

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Differentiation of Argyrophil and Argentaffin Cells in Organotypic Cultures of Embryonic Chick Intestine

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WITH TWO PLATES

INTRODUCTION

THE problem of the differentiation of enterochromaffin cells in the isolated intestine of the chick was first tackled by Simard & van Campenhout (1932) and by Ghidini (1940) by means of chorioallantoic grafts. They showed that enterochromaffin cells, demonstrated by argentaffin methods, differentiate in the grafts in the same way as they do in normal developmental conditions.

These results disproved any passage of enterochromaffin substance (enteramine) from the lumen of the gastro-intestinal tube, but still left the question open as to whether this substance is synthesized in the enterochromaffin cells themselves or whether it reaches them through the blood circulation from other sites of the organism. In the latter case the intestinal wall would work merely as an organ of deposition or excretion of circulating enteramine. Organotypic cultures of intestine, in which the organ is completely isolated from any humoral connexion with the whole organism, may be the means of solving the problem concerning the ability of intestinal cells to elaborate the substance in question.

In this connexion other problems referring to the histogenesis of enterochromaffin cells may be considered, such as: (1) whether they are especially differentiated epithelial cells of endodermic origin, as is believed by most authors (Parat, 1924 *a*, *b*; Cordier, 1926; Simard & van Campenhout, 1932; Vialli & Erspamer, 1936; Erspamer, 1954; Vialli, 1954) or whether they are formed in the connective tissue and then migrate into the epithelium, as claimed by Kull (1913, 1925) and Dias-Amado (1925 *a*, *b*); and (2) whether the same succession of stages as has been shown to occur in normal developmental conditions from argyrophil pre-enterochromaffin to argentaffin enterochromaffin stage takes place *in vitro*. This question is discussed in another paper by the present author (Monesi, 1960). The two stages mentioned above are also believed, on theoretical

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grounds, to occur during elaboration of enteramine throughout life (Erspamer, 1937, 1938, 1954; Vialli & Erspamer, 1939; Vialli, 1954).

In order to know whether the enterochromaffin cells can differentiate in the isolated intestine cultivated *in vitro*, it is essential first of all to determine the developmental stage in which no such cells are present in the material to be used for the cultures. The experimental research *in vitro* was therefore preceded by a preliminary systematic study of the intestine of chick embryos at different ages, at intervals of 24 hours, from the 8th day of incubation until hatching, so as to establish the age and the sites in which enterochromaffin cells appear. The details of this research are published elsewhere (Monesi, 1960) and only the conclusions are given here. The first argentaffin cells, demonstrable with the Masson–Hamperl method, appear in the intestine of the chick embryo at the 15½–16th day of incubation in two places, the duodenum and the umbilical loop near its apex. In later stages they spread to the whole of the small intestine. Argyrophil cells, positive by the Bodian method, differentiate at an earlier stage and may be found as early as the 14th day, in the same parts of the intestine. Consequently, between the 14th and the 15½–16th day, the enterochromaffin system is represented only by argyrophil cells. This supports the theory put forward by Erspamer (1937, 1938, 1954), Vialli & Erspamer (1939), and Vialli (1954) that the argyrophil, non-argentaffin granules are the forerunners of the typical enterochromaffin ones, which combine both characteristics, being at the same time argyrophil and argentaffin. For a short period, beginning with the appearance of the argentaffin cells, i.e. at the 16th and 17th day of incubation, argyrophil cells show a remarkable prevalence over argentaffin cells, as they are four times as numerous as the latter. In more advanced stages the numerical relationship comes down to about 2:1, and remains so throughout life. Most of these cells, both argyrophil and argentaffin, are situated from their first appearance in the epithelium: only a very small number of them may be found, with the same frequency at any age, in the connective tissue of the lamina propria.

MATERIAL AND METHODS

Pieces of intestine of chick embryos, from the 9th to the 19th day of incubation, were cultured *in vitro*. From what has been said above, the stages until the 13th day doubtless precede the first appearance of argyrophil (14th day) and argentaffin cells (15½–16th day). The experiments made with embryos older than the 13th day were expected to show the behaviour of the argyrophil (14th–15th day) or of the argentaffin (16th day onwards) cells already in existence.

The parts of the intestine chosen for the cultures were the descending parts of the duodenum and the summit of the umbilical loop. These parts, besides being easy to identify, correspond to the sites where enterochromaffin cells begin to differentiate, and therefore offer a better chance of positive results.

The embryos belonged to the same breeds (New Hampshire and Rhode Island) used for the preliminary *in vivo* research already quoted. However, to allow for individual variability in the age of appearance of enterochromaffin cells, control tissues were taken, at the moment of explantation, from all embryos employed for the cultures; these control tissues were obtained from the intestine immediately contiguous to the piece which was used for culturing. The sites of controls and cultures were alternatively exchanged.

The technique employed was to culture on the surface of a clot, in a watch-glass contained in a Petri dish, according to Fell & Robison (1929). The clot consisted of 5 drops of a non-heparinized adult cock plasma and 5 drops of extract from 9-day chick embryos. Some cultures were made in embryonic extract only in order to avoid the possibility that some enterochromaffin substance might reach the tissue through the plasma contained in the medium.

From each embryo, and from each of the two parts of the intestine selected, 3–5 cultures were made depending upon the size of the embryos. The cultures were kept in an incubator at 37° C. for 2–6 days, and the medium was changed every 2nd day. It was not possible to continue cultivation beyond 6 days because of the constant appearance of general degeneration.

At the end of the experiments all cultures were washed in Tyrode and fixed, some in 10 per cent. neutral formol, others in a mixture of ethyl alcohol (90 parts), formol (5 parts), and acetic acid (5 parts). In addition, to provide a general idea of the pattern of the cultures, a few sections were stained in haematoxylin-eosin, and some were fixed for this purpose in Susa fluid.

The histochemical research on enterochromaffin cells was carried out by means of two methods: (1) the argentaffin reaction of Masson–Hamperl, after fixation in formol, which according to Erspamer (1937, 1938, 1954), Vialli & Erspamer (1939), and Vialli (1954) demonstrates the typical enterochromaffin cells. The immersion in ammoniacal silver nitrate always lasted 24 hours; and (2) the argyrophil reaction of Bodian after either of the two fixations mentioned above. This shows both typical enterochromaffin cells and argyrophil pre-enterochromaffin ones. The modification suggested by Dawson & Barnett (1944) was adopted, which consists in repeating twice the treatment with protargol and the subsequent reduction. Moreover, at the end of the second impregnation, and before reduction, the preparations were taken out of the incubator at 37° C. and put into another incubator at 60° C. for 4 hours. This treatment is specially useful for the cultures of intestine of young embryos, in which argyrophil granules are very difficult to impregnate.

The difference between the number of Bodian positive argyrophil cells and Masson positive argentaffin ones should indicate the number of pre-enterochromaffin cells. However, Hamperl (1952) and Hellweg (1952) claim that a small part, about 5 per cent. of the total enterochromaffin system, consists of only argentaffin, non-argyrophil cells.

It is generally admitted (Vialli & Erspamer, 1939; Erspamer, 1954; Vialli, 1954) that the two types of cells belonging to this system do not represent cellular categories, but successive stages of the same secretory cycle. In other words, pre-enterochromaffin, argyrophil granules represent an early step in the synthesis of the enteramine, which ends with the argentaffin stage. However, this conclusion cannot be completely generalized. In fact, in some conditions (Erspamer, 1937; Vialli & Erspamer, 1939; Dawson, 1948; Dawson & Moyer, 1948; Vialli, 1954) the enterochromaffin system is represented by argyrophil cells only, so that this stage is the final one.

RESULTS

Cultures of intestine of 9- and 10-day embryos kept their normal structure, even after 6 days of culture, and went on differentiating morphologically and cytologically. The villi, which had just begun to form when the culture was begun (Plate 1, fig. 1), increased in number and reached their definitive shape during the period of cultivation (Plate 1, fig. 2). It may be remembered that formation of villi *in vitro* was observed also by Chlopin (1922) in explants of intestine of rabbit embryos.

Cultures of intestine explanted in more advanced stages from the 12th day onwards with villi which had already reached a considerable development (Plate 1, fig. 3), when transversely cut as small portions of tubes, preserved their original structure fairly well at the ends of the explants, from which the villi tended to protrude; but the central zone of the cultures, especially in the part immediately touching the clot, degenerated very rapidly and became grossly necrotic as early as 4 days after explantation. To avoid this, a different technique was used for most cultures made from these stages. A piece of intestine, after being transversely cut, was divided longitudinally into two halves. These, through muscular contraction, rolled up with the epithelium turned outward. In this case also the immediate contact with the plasma had an unfavourable effect on the preservation of the villi, which disappeared, while the epithelium flattened, as Fischer (1922) had noted in explants of intestine taken just before hatching. But all the rest of the surface which remained free preserved its villi, although these changed their shape to a greater or lesser extent, the more remarkably so the older the stage at explantation. The most frequent alteration was a rarefaction and swelling of the stroma (Plate 1, fig. 4) which, however, did not affect the structure of the epithelium and, in any case, was not general, because even in these cases quite normal-shaped villi were found (Plate 1, fig. 5).

In any case, the epithelium appeared to be the most resistant structure in the cultures, and although in some parts it was flattened it preserved its normal shape in many areas of all cultures so as to allow, with appropriate methods, recognition of the enterochromaffin cells, which are the main object of the present research.

The cultivated fragments often showed slow muscular contractions similar to that observed and widely discussed in many papers concerning cultures of intestine and stomach of chick embryos (Fischer, 1922; Bisceglie, 1932; Gozzi, 1940; De Jong & De Haan, 1943; Keuning, 1948).

The results concerning the time of appearance and the general behaviour of the cells giving argyrophil and argentaffin reactions are summarized in Table 1. For each embryonic stage figures are given of the number of embryos used, the number of all cultures made, and the number of those fixed in formol at different times. For the sake of simplicity, cultures fixed at 2 and 3 days, and at 4 and 5 days, were grouped together. All cultures were serially sectioned and stained in different sections for both argyrophil and argentaffin cells so as to allow cultures of any age to be listed as negative, argyrophil positive, and argentaffin positive. It will be clear from what follows that the second and third items of this classification coincide almost completely, so that the total number in each line of any group corresponds to negative cultures plus the highest number entered in one of the positive ones.

The last column includes all cultures not utilizable from this point of view, whether accidentally lost after fixation or infected or totally degenerated, or fixed in other ways than in formol.

In no culture of intestine of 9-day chick embryos did argyrophil or argentaffin cells develop, even after 6 days of culture.

Cultures of intestine of 10-day embryos, containing no argyrophil or argentaffin cells at the time of explantation, developed neither of these cells in 2–5 days; but after 6 days of cultivation, two cultures showed the presence of a small number, 4–5 in each culture, of argentaffin cells (Plate 2, fig. 6). In one of these cultures a few cells giving the argyrophil reaction were also found.

In cultures of intestine made from 12- and 13-day-old chick embryos, which therefore still belonged to a stage at which both argyrophil and argentaffin cells are absent, these cells first begin to appear after 4 days *in vitro* (Plate 2, figs. 7, 10). At the 5th and 6th day of culture their number has remarkably increased. Some of them are illustrated in figs. 8, 9, and 11 of Plate 2.

No regular appearance of argyrophil cells before the argentaffin cells was ever observed in these cultures. No general rule can be given about the frequency of the silver-impregnated cells, as their number varies remarkably from culture to culture and also in different sections of the same culture according to the state of health of the epithelium. In the most favourable cultures, fixed 4 days after explantation, the number of argentaffin cells can be from 0 to 6 in each 7-μ-thick transverse section. The number of argyrophil cells can be equal or slightly, but not significantly, superior. No attempt was made to do a systematic count of the cells, as it would not give reliable results because of the different state of preservation of the histological structure of the explants. It may be safely stated, however, that both argyrophil and argentaffin cells differentiated *in vitro* are always fewer than those which develop *in vivo* in the same time. Also, the number of silver-impregnated granules found in the cells of the cultivated intestine is smaller than that found in the whole embryo in normal

TABLE 1

Number of cultures fixed in formol

Age of the embryos (days)	*Number of embryos used*	*Total number of cultures made for each age*	*After 2–3 days*				*After 4–5 days*				*After 6 days*				*Number of cultures lost or fixed otherwise than in formol*
			Containing neither argyrophil nor argentaffin cells	*Containing argyrophil cells*	*Containing argentaffin cells*	*Total number*	*Containing neither argyrophil nor argentaffin cells*	*Containing argyrophil cells*	*Containing argentaffin cells*	*Total number*	*Containing neither argyrophil nor argentaffin cells*	*Containing argyrophil cells*	*Containing argentaffin cells*	*Total number*	
9	10	43	2	. .	. .	2	17	. .	. .	17	19	. .	. .	19	5
10	4	52	4	. .	. .	4	12	. .	. .	12	28	1	2	30	6
12	3	36	7	. .	. .	7	4	4	6	10	2	6	9	11	8
13	4	44	8	. .	. .	8	7	12	11	19	2	7	11	13	4
14	3	54	12	7	7	19	3	20	18	23	. .	6	5	6	6
15	3	60	7	8	14	21	6	12	15	21	. .	8	10	10	8
16	2	20	. .	10	10	10	. .	4	4	4	. .	. .	. .	. .	6
17	1	15	. .	8	8	8	. .	5	5	5	. .	. .	. .	. .	2
19	1	12	. .	10	10	10	. .	. .	. .	. .	. .	. .	. .	. .	2

conditions. In the cultures, the granules are more frequently limited to the basal pole of the cells, and the number of cells containing granules throughout their cytoplasm is much smaller.

Most argyrophil and argentaffin cells which have developed *in vitro* make their first appearance in the epithelium, i.e. they are found in the same situation as *in vivo*. Only very few are situated in the connective tissue of the lamina propria.

In cultures from 14- and 15-day embryos, belonging to a stage in which a few argyrophil cells are already present at explantation, numerous argyrophil and argentaffin cells were found after only 2 days of cultivation. The number of the former increased in culture; the latter were formed during cultivation. In 4 days' time the number of argentaffin cells in a transverse 7-μ-thick section ranged from 2 to 10 when the cultures came from 14-day embryos, and from 5 to 20 when they came from 15-day-old embryos. The number of argyrophil cells is more or less equal, or perhaps very slightly higher than that of the argentaffin cells. The number of both increased remarkably after 4 and 5 days of cultivation. The total amount of granules of both types increased when compared with the cultures of intestine of 12- and 13-day-old embryos; the number of cells containing granules throughout the cytoplasm also increased.

Embryos of 16, 17, and 19 days contain numerous argyrophil and argentaffin cells; in cultures of these stages the number of both types of cells appears to increase during cultivation.

In order to avoid the possibility that some enteramine contained in the adult cock plasma used for the medium was the source of the enterochromaffin granules differentiated during cultivation, some cultures were prepared using as a medium only extract made from 9-day-old embryos. Although the behaviour of the cultures was less favourable, the appearance of silver impregnable cells was observed.

DISCUSSION

The present research shows that enterochromaffin cells differentiate in the intestine of the chick embryo cultivated *in vitro*. They appear in organotypic cultures of intestine explanted from 10-, 12-, and 13-day-old embryos, belonging to stages before the first argyrophil and argentaffin cells are produced. The two cell types appear *in vivo* only at the 14th and 15½–16th day respectively. The absence of such cells at explantation time was proved not only by a systematic study of other embryos of the same breed and age, incubated with those used for the experiment, but also by a systematic study of a strictly contiguous zone of intestine from the same embryos used for the cultures. In cultures from embryos of later stages the argyrophil cells, present from the 14th day onwards, and the argentaffin cells, first appearing at the 15½–16th day, increased in number during the period of cultivation.

However, the total number of both types of cells differentiating *in vitro* is evidently always smaller than that found in normal conditions at the same total age *in vivo*, and the number of granules is also inferior.

In culture, no stage was found in which argyrophil cells only are present; this is contrary to what occurs *in vivo* where, as shown elsewhere (Monesi, 1960), argyrophil cells precede the appearance of argentaffin cells by two days. It cannot, however, be concluded that the usual succession from the argyrophil, pre-enterochromaffin stage to the final argentaffin or enterochromaffin proper, does not take place in culture. It may be suggested that the passage from the first to the second stage occurs *in vitro* more quickly than *in vivo*, so that the former is unlikely to be observed. This assumption is perhaps supported by the observation that in culture, the frequency of argyrophil cells is more or less the same or very slightly higher than that of the argentaffin cells, whereas *in vivo* it is 3 or 4 times as much in the early stages of their differentiation, and comes down to twice in more advanced stages and at hatching.

As *in vivo*, most enterochromaffin cells, either argyrophil or argentaffin, which differentiate in culture are situated from their first appearance in the epithelium, occupying its whole thickness from the basement membrane to the intestinal lumen. The impression that one sometimes gathers, both *in vivo* and *in vitro*, that some are outside the epithelium, probably derives from the fact that the nuclei of enterochromaffin cells are often at a lower level than those of the remaining epithelial cells (Plate 2, figs. 10, 11). The observations by Masson (1928) and Feyrter (1953) seem also to confirm this tendency of enterochromaffin cells to bulge towards the lamina propria while remaining above the basement membrane.

However, apart from that, in the cultures as well as in natural developmental conditions a small number of cells containing argyrophil or argentaffin cells were found outside the epithelium, i.e. in the connective tissue of the lamina propria. This finding does not depend upon tangential sections of the epithelium. As *in vivo*, in no case is there a stage in which the enterochromaffin cells have, exclusively or prevalently, an extra-epithelial situation: the first argyrophil or argentaffin cells which differentiate in culture are mostly in the epithelium, very few being in the connective tissue; the frequency of the latter does not change when cultivation is prolonged. It may therefore be concluded that, like the results obtained in the study of development *in vivo* (Monesi, 1960), the results from cultures disprove the theory of Kull (1913, 1925) and Dias-Amado (1925 *a*, *b*) that enterochromaffin cells differentiate in the mesenchyme and subsequently migrate into the epithelium. On the contrary, it is more likely that extra-epithelial enterochromaffin cells are, as suggested by Simard & van Campenhout (1932), originally intra-epithelial cells which then wander into the connective tissue. Of course, all these considerations refer only to the cells when they are identifiable through specific methods: it cannot be excluded that in the differentiation of enterochromaffin cells an earlier stage may exist in which they do not contain granules, or in which the granules do not give the characteristic reactions.

The differentiation of enterochromaffin cells as shown to take place in the isolated intestine cultured *in vitro* gives substantial support to the theory of the synthesis inside the intestinal cells of the enterochromaffin substance, or enteramine. Experiments with chorioallantoic grafts (Simard & van Campenhout, 1932; Ghidini, 1940) proved that this substance is not derived from the lumen of the gut. The fact (Simard & van Campenhout, 1932) that the cells in question appear in grafts made from a stage preceding that of the establishment of the normal nervous connexions (92nd hour of incubation according to van Campenhout, 1931) is against the theory put forward by Danisch (1924) that these cells are nervous or formed under the influence of the nervous system.

The present research, which separates the intestine from the influence of the blood circulation, proves that the enteramine is elaborated by the enterochromaffin cells themselves and does not derive from other parts of the organism, and that the enterochromaffin system cannot be considered as an organ of deposition or excretion of enteramine. Nor can it be believed that enteramine contained in the plasma used as culture medium is deposited in the cultivated intestine. Apart from the fact that the plasma content of enteramine is in any case extremely low, and probably zero according to the most recent work (Erspamer, 1954), it should be noted that enterochromaffin granules were observed also in cultures in which the medium consisted only of extract of chick embryos of 9 days of incubation.

SUMMARY

1. Argyrophil, Bodian positive cells, and argentaffin, Masson–Hamperl positive cells differentiate *in vitro* in organotypic cultures of intestine of chick embryos explanted before argyrophil and argentaffin cells are known to differentiate, which is at the 14th and the 15½–16th day of incubation, respectively.

2. During culture *in vitro*, argyrophil and argentaffin granules differentiate simultaneously; the number of cells demonstrable with either method does not show any significant difference throughout the duration of the cultures, which were incubated up to a maximum of 6 days. This result is at variance with what occurs *in vivo*, where argyrophil cells differentiate earlier than argentaffin cells and are in earlier stages 3 or 4 times as frequent as the latter, becoming only twice as numerous at a later stage.

3. As in development *in vivo*, argyrophil and argentaffin cells differentiating in the intestine of the chick embryo are mainly situated in the epithelium. Only very few are found in the connective tissue of the lamina propria.

4. Simard & van Campenhout (1932) and Ghidini (1940) showed that enterochromaffin granules differentiate in chorioallantoic grafts of intestine of chick embryos and they disproved in this way any kind of derivation of the enterochromaffin substance (enteramine) from the lumen of the gastrointestinal tract, as well as its formation under the influence of normal nervous connexions. It is now shown that the specific granules differentiate also in intestine cultivated *in*

vitro and therefore in conditions which exclude any other possible mode of correlation with the whole organism, and particularly any uptake of enteramine from the blood, thus strongly supporting the conclusion that it is synthesized *in situ*.

RIASSUNTO

La differenziazione di cellule argentofile e argentaffini in culture organotipiche di intestino embrionale di pollo

1. Cellule argentofile, positive al metodo Bodian, e cellule argentaffini, positive al Masson-Hamperl, si differenziano *in vitro* in culture organotipiche di pezzi di intestino di embrione di pollo, espiantati prima dell'età in cui compaiono le prime cellule argentofile e argentaffini, cioè rispettivamente al 14° giorno, e al $15\frac{1}{2}$–16° giorno di incubazione.

2. In cultura i granuli argentofili si differenziano contemporaneamente agli argentaffini; il numero di cellule dimostrabili con l'uno e con l'altro metodo non dimostra alcuna differenza significativa per tutta la durata delle culture, che giunse fino a un massimo di 6 giorni. Questo risultato differisce da ciò che avviene *in vivo*, dove le cellule argentofile compaiono più precocemente delle argentaffini e rimangono negli stadi più precoci tre o quattro volte più numerose di queste ultime, mentre in stadi più avanzati si riducono al doppio.

3. Similmente a ciò che avviene *in vivo*, le cellule argentofile e le argentaffini si differenziano nell'intestino di pollo prevalentemente nell'epitelio. Soltanto un piccolo numero si trovano nel tessuto connettivo della tonaca propria.

4. Simard & van Campenhout (1932) e Ghidini (1940) dimostrarono che i granuli enterocromaffini si differenziano negli innesti corioallantoidei di intestino di embrioni di pollo, escludendo in tal modo che la sostanza enterocromaffine o enteramina possa derivare dal lume del tubo gastroenterico, come pure la sua formazione sotto l'influenza delle normali connessioni nervose. Nella presente ricerca viene ora dimostrato che i granuli specifici compaiono anche nell'intestino coltivato *in vitro*, dunque in condizioni tali da escludere ogni altra possibile correlazione con l'intero organismo e in particolare la provenienza dell'enteramina attraverso la circolazione sanguigna, così da aggiungersi un forte argomento in favore della sua sintesi *in situ*.

ACKNOWLEDGEMENTS

Thanks are due to Professor E. Borghese and Professor M. Vialli for their many helpful suggestions and criticisms and to Dr. E. Vivori for her very kind help with the English text.

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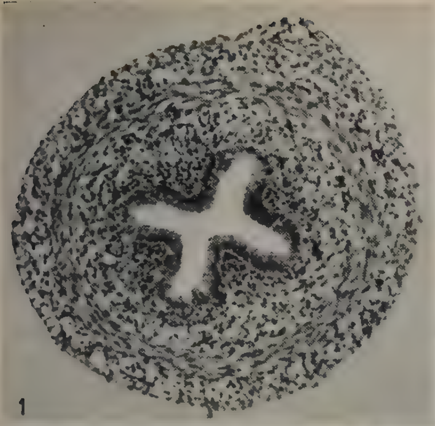
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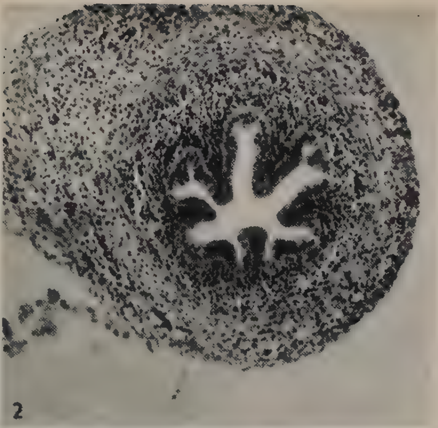
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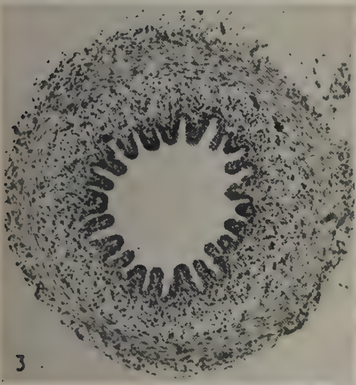
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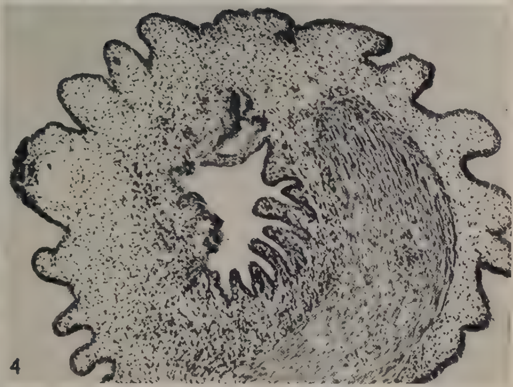
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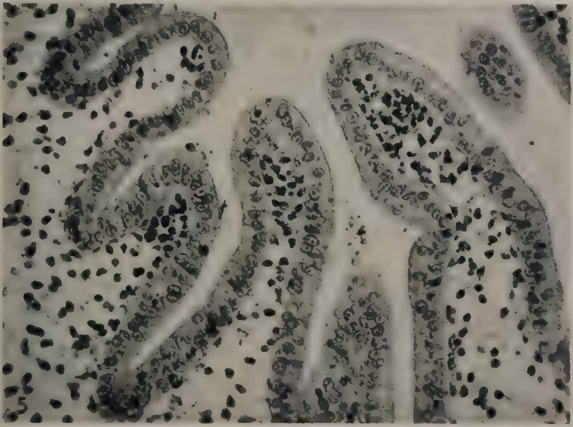
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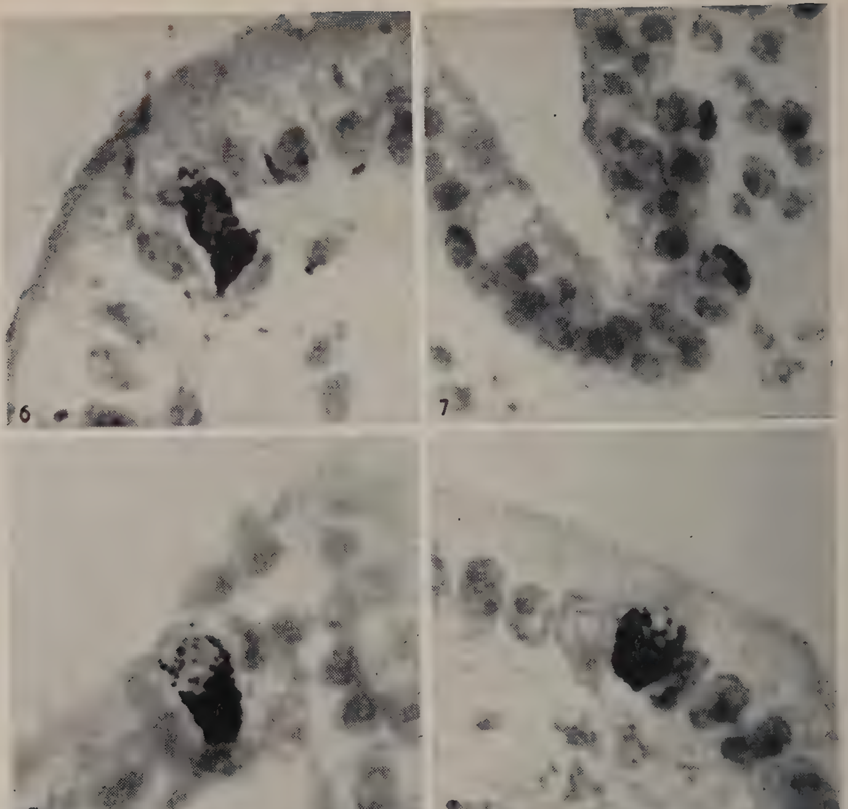
V. MONESI

Plate 1



V. MONESI

Plate 2

VIALLI, M., & ERSPAMER, V. (1936). Contributo alla migliore conoscenza istochimica delle cellule enterocromaffini. *Arch. ital. Anat. Embriol.* **37**, 411–36.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Intestine of a 9-day chick embryo, near the apex of the umbilical loop, showing a few villi just forming. Formol; haematoxylin and eosin. × 165.

FIG. 2. Intestine of a 9-day chick embryo, near the apex of the umbilical loop, cultivated for 5 days. The villi have increased in number and taken a more definite shape. Formol; carmalum. × 165.

FIG. 3. Intestine of a 12-day chick embryo, near the apex of the umbilical loop, showing numerous villi. Formol; haematoxylin and eosin. × 90.

FIG. 4. Intestine of a 12-day chick embryo, near the apex of the umbilical loop, longitudinally cut when explanted and cultivated for 6 days. In spite of great morphological deformation, almost normal villi are still present in some parts of the culture, as in the centre of the figure; most of them are short and broad: the epithelium is consistently normal. Formol; haematoxylin and eosin. × 90.

FIG. 5. Intestine near the apex of the umbilical loop, explanted at the 13th day and cultivated for 5 days. A detail showing very good preservation of the villi with their epithelium. Alcohol-formol-acetic acid; haematoxylin and eosin. × 295.

PLATE 2

FIG. 6. Piece of umbilical loop explanted at the 10th day of incubation and cultivated for 6 days. An argentaffin cell, which appeared during culture. Formol; Masson–Hamperl. × 1950.

FIG. 7. Piece of umbilical loop explanted at the 12th day of incubation and cultivated for 4 days. Two cells showing argentaffin granules at their bases, which appeared during culture. Formol; Masson–Hamperl; Carmalum. × 430.

FIG. 8. Piece of umbilical loop explanted at the 12th day of incubation and cultivated for 6 days. An argentaffin cell which appeared during culture. Formol; Masson–Hamperl. × 1950.

FIG. 9. Piece of umbilical loop explanted at the 12th day of incubation and cultivated for 6 days. An argyrophil cell which appeared during culture. Formol; Bodian. × 1950.

FIG. 10. Piece of duodenum explanted at the 13th day of incubation and cultivated for 4 days. An argyrophil cell which appeared during culture. Formol; Bodian. × 1950.

FIG. 11. Piece of duodenum, explanted at the 13th day of incubation and cultivated for 5 days. An argyrophil cell which appeared during culture. Formol; Bodian. × 1950.

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The Inhibitory Action of Antimycin A in the Early Chick Embryo

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WITH ONE PLATE

ANTIMYCIN A, an antibiotic obtained from an undetermined species of streptomyces, was isolated, crystallized, and described by Dunshee, Leben, Keitt, & Strong (1949), and its biological action has been studied by many workers since then. Ahmad, Schneider, & Strong (1950) demonstrated its effects on the growth and metabolism of yeast, on enzyme activities in the succinoxidase system, and on rats given the drug orally. Potter & Reif (1952) confirmed the inhibitory effect of antimycin A on the succinoxidase system in liver, suggested the presence of an 'antimycin A-blocked factor', identical, probably, with the 'Slater factor' and showed that, in certain tissues, there is an antimycin A-insensitive pathway for DPN oxidation. The same workers, Reif & Potter (1954), used the drug to characterize the pathways of DPN oxidation in different tissues. Green, Mii, & Kahout (1955) and Thorn (1956) argue from their experiments that the BAL-sensitive (Slater) factor and the antimycin A-sensitive factor are not identical.

Duffey & Ebert (1957) employed antimycin A as an experimental tool in the investigation of developing tissues in the chick embryo. Using the explantation technique of Fell & Robison (1929) as modified by Spratt (1947) they showed that, like sodium fluoride, antimycin A produced a strong inhibitory effect on the heart-forming mesoderm resulting in complete absence of the heart; somites formed in the presence of the drug but did not persist, otherwise the embryos showed normal development.

In the work described here, the experiments with antimycin A as a metabolic inhibitor were extended to the use of the explantation technique described by New (1955) and the results show that tissues other than mesodermal derivatives may be affected. The location of the abnormalities produced appears to depend on the precise method of application of the drug.

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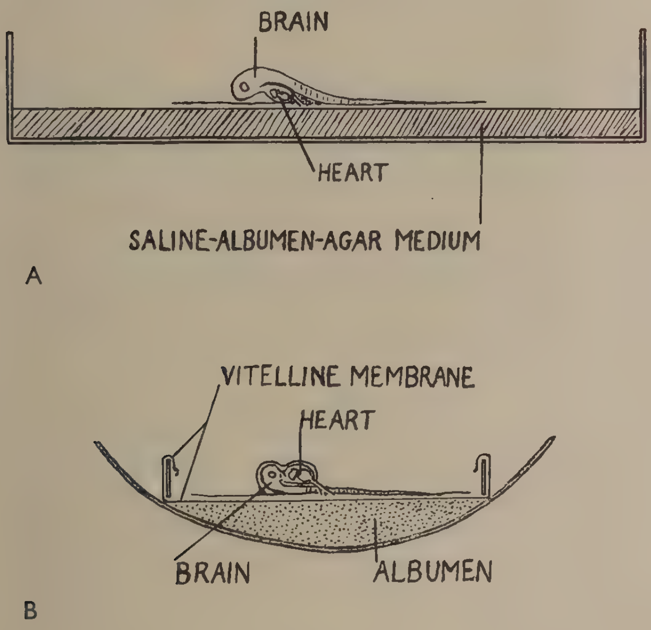
MATERIALS AND METHODS

All the eggs were from pure White Leghorn stock, and the drug, obtained from the Wisconsin Alumni Research Foundation, was stored as a stock solution (20 mg. in 200 ml. of 95 per cent. ethanol) and diluted for use with Howard–Ringer (Howard, 1953) saline. Confirmatory tests were carried out with the Spratt (1947) technique (Text-fig. 1A) as used by Duffey & Ebert (1957). The New (1955) technique (Text-fig. 1B) was employed for most of the experiments, but the method of introducing the antimycin A varied; in some cases it was mixed with the albumen underlying and applied to the dorsum of the embryo through the vitelline membrane, and in others it was placed on the ventral surface of the disk. A series of experiments was also conducted in which the antimycin A was administered *in ovo*, either into the albumen or into the yolk.



TEXT-FIG. 1

Eggs were incubated at 37° C. initially for a period between 18 and 22 hours to bring the embryos to stages 4–9 (Hamburger & Hamilton, 1951) and subsequently with antimycin A for 18–24 hours.

RESULTS

Repetition in detail of the work of Duffey & Ebert (1957) confirmed their results. 0·025 γ antimycin A was added to each millilitre of the saline–albumen–agar medium. Of the 21 specimens explanted under these conditions at stages 5–8, none showed heart formation during the next 24 hours' incubation: where somites were present they were irregular and poorly developed. In 14 specimens the central nervous system was normal, in the remainder poorly developed or distorted.

In the first group of experiments using the New technique, the antimycin A, diluted from the stock solution with Howard–Ringer saline, was added to the nutrient medium (albumen). With a dose of 0·03 γ or more for each specimen, there remained no sign of an embryo after 20–24 hours; where damage was less severe and dead, distorted material persisted, lysis of tissue was often marked in the region of the primitive streak. Thirty-four embryos each received 0·025 γ antimycin A; 10 embryos were recovered showing no gross abnormality, 1 had completely disintegrated, and the remainder displayed moderate or gross distortion of the brain often accompanied by lysis of tissue around the central axis and anomalies of the somites. Cardia bifida was recorded once, the only malformation of the heart in this series. Figs. A and B of the Plate illustrate typical findings, and fig. C shows necrosis of neural tissue and of the dorsal part of the somite in a specimen treated in this way.

The pattern of abnormalities obtained when 0·01–0·013 γ antimycin A was applied to the ventral surface of the embryo was quite different. Treatment of specimens which had reached only stages 4 or 5 often caused general inhibition or cessation of growth during the next 18–20 hours, but where development had been less affected it was found that the heart-forming tissue was more seriously damaged than brain, e.g. Plate, figs. D, E: note the relatively healthy appearance of the somites. If the embryos had reached stages 6–8 when explanted, a significant increase in the number of normal specimens was found after 20 hours' incubation with the same dose of antimycin A. Normal, or almost normal, hearts and brains, however, were often accompanied by severe damage to the somites; embryos were recovered showing virtually complete mesodermal degeneration caudal to the heart and brain while, in some, only a transverse segment of somite-forming material was absent, normal tissue existing anterior and posterior to the damaged area (Plate, fig. F). The last-mentioned finding was correlated with and emphasized by the behaviour of one specimen explanted at stage 4 and treated with antimycin A on its ventral surface; after 18 hours it was noted that, although 5 somites were present, no heart had formed and the neural plate appeared healthy. Following a further 4 hours' incubation on the same medium, there was still no heart but 9 somites were now visible and the brain had progressed to the formation of the optic vesicles.

Treatment of embryos *in ovo* is unsatisfactory compared with *in vitro* techniques where the initial developmental stage can be determined accurately and the drug located precisely, but a small series of experiments was carried out varying the dose of antimycin A from 0·1 to 1·0 γ for each egg, the injection being made either into the albumen or into the yolk. It was not possible, however, to decide whether the variation in the site of the injection played a significant part in the type of abnormality produced. Although anomalies of the nervous system were prominent after albumen or yolk injection, they were more frequent in the former; similarly, more of the recorded cardiac abnormalities were in the series of yolk injections. Many specimens, however, showed both types of defect and, especially with the higher dosages, inhibition of growth or even death was common.

DISCUSSION

Extensive investigation of antimycin A since its discovery in 1949 has established it as a strong metabolic inhibitor with a specific effect on the succinic oxidase system.

The 2,3-dimercaptopropanol (BAL) sensitive factor (Slater factor) in the oxidation of reduced DPN was identified as its probable site of action by Potter & Reif (1952), while Thorn (1956) postulated a separate antimycin A-sensitive factor. Such specificity might be expected to prove useful in the investigation of tissue metabolism, and indeed Reif & Potter (1954), using heart, kidney, liver, and Flexner–Jobling carcinoma, demonstrated a differential inhibition of respiration in these tissues.

Employing the chick embryo cultured *in vitro*, Spratt (1950) reported that nervous tissue, dependent on enzymes catalysing respiration, is more easily inhibited by oxygen deficiency, cyanide, and iodoacetate, while heart, dependent chiefly on glycolysis, is more sensitive to fluoride. By the same explantation technique, Duffey & Ebert (1957) compared the actions of fluoride and antimycin A and demonstrated an effect which was specific and, except for minor differences, similar for both these substances on the heart-forming mesoderm and somites. Thus antimycin A, a drug known to exert its action on a respiratory mechanism, was found to inhibit, with remarkable specificity, a tissue credited with a considerable dependence on glycolytic mechanisms.

The experiments described here show that the effect of the antimycin A varies with the technique employed; where the drug has been applied to the ventral surface of the embryo, the mesoderm is inhibited with little or no damage to the neural tube; if it is presented to the embryo from the dorsal aspect, brain tissue and the dorsal part of the somite suffer inhibition and often necrosis.

Comparison of the effects of antimycin A at different stages of development in the chick are interesting and important. Applied at stages 4 or 5, it may yield acardiac embryos with normal somites, whereas if applied at later stages, in which the primitive heart may have already appeared, it can destroy or inhibit somite formation without interference with heart or brain. Furthermore, there may be somite damage localized geographically and, presumably, chronologically as well (Plate, fig. F). Two conclusions can be drawn from these observations: first, that there must be, for heart and for somites, phases of differentiation when each is more susceptible to the action of antimycin A, such phases occurring at or about stage 4 for the heart and over a series of later stages for the somites; and second, that a process of inactivation of antimycin A must occur as a result of its toxic effect on the tissues, allowing normal growth and differentiation to occur subsequently. Neither deduction is original. It has long been recognized not only that organs and tissues experience their initial phases of activity at different stages but also that they are more susceptible to the injurious action of both chemical and physical agents at these times. In regard to the second, Reif & Potter (1953) demonstrated inactivation of antimycin A but stated that it could be reversed by adding serum proteins. Thorn (1956) confirmed the 'pseudo-irreversible' nature of antimycin A by showing that in spite of a strong combination between it and the antimycin A-inhibitable factor, addition of a tissue preparation, in which the succinic oxidase system was specifically and irreversibly inhibited by *p*-aminophenylarsenoxide, caused reactivation. This renewed activity by the drug was probably due to a redistribution of the antimycin A over the increased quantity of susceptible factor and was inversely proportional to the increased ratio of heart-muscle preparation to inhibitor. On this basis, our experiments showing localized defects in somite formation apparently involved doses of antimycin A so critical that reactivation of the drug subsequent to the production of fresh antimycin A-inhibitable factor in the differentiating tissue was not demonstrable because the concentration of antimycin A had fallen below the threshold value for inhibition.

SUMMARY

Antimycin A has been administered to chick embryos at stages 4–9 in a variety of ways following well-established techniques. The pattern of abnormalities obtained depends essentially on the avenue of approach of the drug to the embryo; if it is applied to the ventral surface, heart and mesoderm are inhibited to a greater degree than nervous tissue; if on the dorsal surface, brain and spinal cord suffer more than mesodermal derivatives.

Heart and somites are susceptible to antimycin A at different stages of development, and Thorn's demonstration, using heart-muscle preparations, of the reversible nature of the combination between antimycin A and the antimycin A-inhibitable factor in the succinic oxidase system is in agreement with our observations in the chick embryo.

RÉSUMÉ

L'action inhibitrice de l'antimycine A chez le jeune embryon de Poulet

L'antimycine A a été administrée à des embryons de Poulet aux stades 4 à 9 suivant différents procédés dérivant de techniques bien établies. Le type des

anomalies obtenues dépend essentiellement du mode d'administration de la drogue à l'embryon; si elle est appliquée à la surface ventrale, le cœur et le mésoderme sont inhibés plus intensément que le tissu nerveux; si elle est appliquée à la surface dorsale, le cerveau et la moelle souffrent plus que les dérivés mésodermiques.

Le cœur et les somites sont sensibles à l'antimycine A à différents stades du développement et la démonstration apportée par Thorn, à propos des préparations de muscles cardiaques, de la nature réversible de la combinaison entre l'antimycine A et du facteur inhibiteur de l'antimycine A dans le système de la succino-oxydase, est en accord avec nos observations sur l'embryon de Poulet.

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EXPLANATION OF PLATE

FIG. A. Explanted by the New technique at stage 6; 0·025 γ antimycin A added to albumen; cultured for 20 hours.

FIG. B. Explanted by the New technique at stage 8; 0·025 γ antimycin A added to albumen; cultured for 20 hours.

FIG. C. Transverse section of embryo explanted by New technique at stage 8; 0·02 γ antimycin A added to albumen; cultured for 19 hours; showing necrosis of neural tissue and dorsal part of somite.

FIG. D. Explanted by New technique at stage 5; 0·01 γ antimycin A applied to ventral surface; cultured for 19 hours.

FIG. E. Explanted by New technique at stage 5; 0·01 γ antimycin A applied to ventral surface; cultured for 19 hours.

FIG. F. Explanted by New technique at stage 7; 0·01 γ antimycin A applied to ventral surface; cultured for 20 hours.

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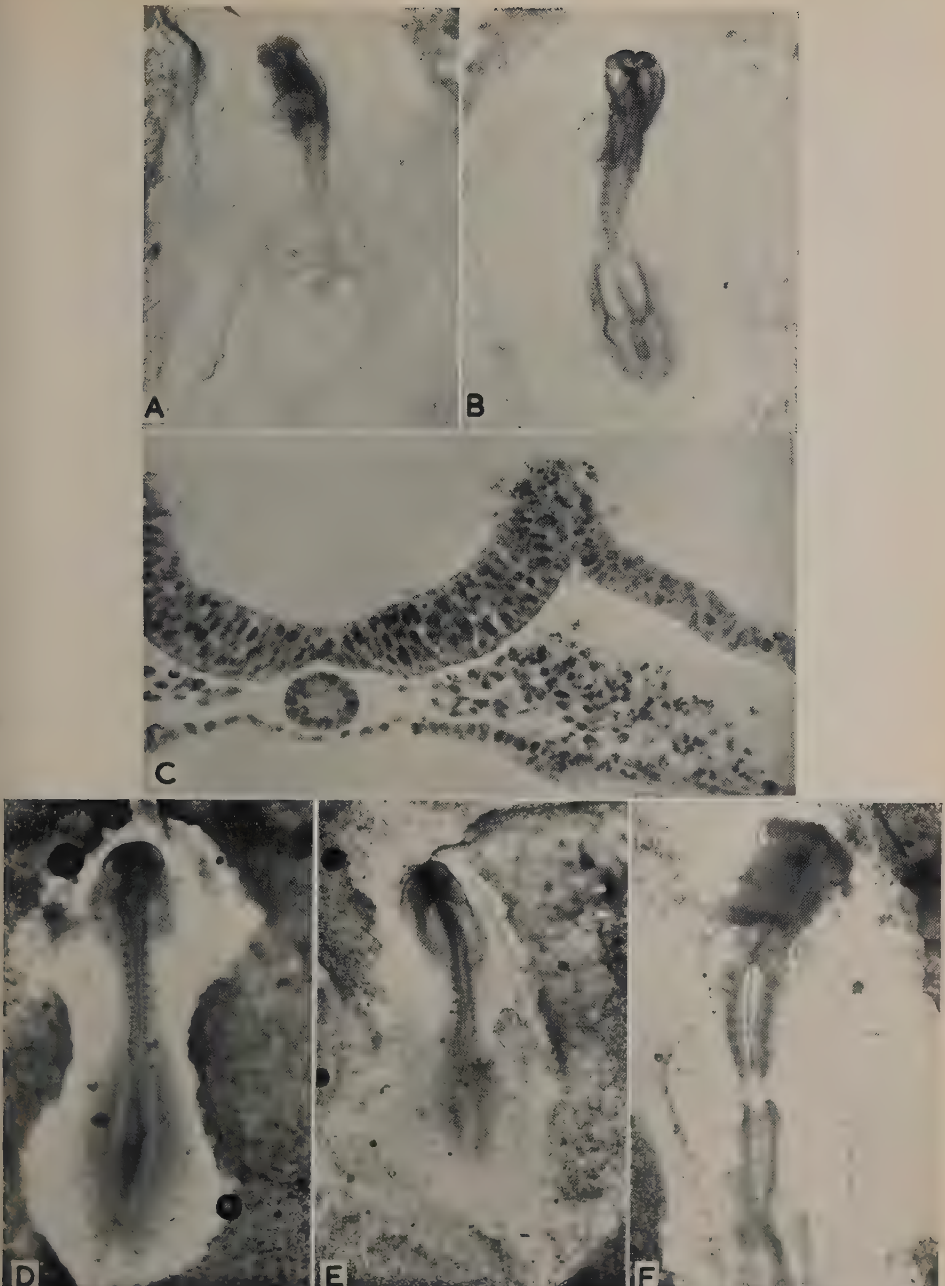

J. McKENZIE *and* J. D. EBERT

Quelques précisions sur la cinématique de la ligne primitive chez le Poulet

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INTRODUCTION

LA formation de la ligne primitive du Poulet a toujours fait l'objet de vives controverses. Les données les plus récentes à ce sujet ont été publiées par Spratt (1942, 1946, 1947), qui a fortement critiqué les méthodes de coloration vitale *in ovo*. Il les remplace par des marques de charbon sur des blastodermes cultivés *in vitro*. Apparemment ses opinions diffèrent fortement de celles de Pasteels (1937). Pour Spratt la zone chordomésoblastique se situe beaucoup plus en avant que Pasteels ne l'indique. En effet, alors que Pasteels la situe au septième postérieur de l'area pellucida du blastoderme non-incubé, elle se trouve pour Spratt d'emblée au centre du blastoderme. Au cours de la gastrulation Pasteels décrit une progression du matériel chordal vers le milieu du blastoderme, tandis que pour Spratt ce matériel chordal déjà en place est atteint par la ligne primitive, qui se creuse d'arrière en avant, dans du matériel qui ne bouge pratiquement pas. Il semble bien que la plupart de ces opinions pourraient, comme l'a essayé Waddington (1952), être rapprochées en tenant compte des techniques utilisées. Une controverse demeure cependant ouverte, celle qui concerne la valeur de la jeune ligne primitive: peut-on en effet admettre avec Pasteels que l'extrémité antérieure de la toute jeune ligne primitive est formée de matériel qui représente le futur nœud de Hensen, ou doit-on le nier, comme le fait Spratt?

MATÉRIEL ET TECHNIQUE

Malgré la réplique de Pasteels aux objections de Spratt, à l'occasion de la publication du mémoire de Malan (1953), le point nous semblait suffisamment important pour essayer de vérifier l'hypothèse de Spratt au moyen de sa propre technique. A cette fin nous avons effectué *in vitro* des sections frontales de jeunes blastodermes de Poulet, exactement en avant de l'extrémité antérieure de l'ébauche de la jeune ligne primitive. Nous n'avons pas utilisé des stades antérieurs à l'apparition de cette ébauche parce que, malgré le grand nombre de critères dont on peut se servir pour orienter un jeune blastoderme à ce stade, le

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risque de se tromper demeure considérable. Les 4 cas (sur 72 cas examinés) où Spratt (1942, p. 76) avoue s'être trompé de 90° sur l'orientation de l'axe du blastoderme ne semblent pas l'inquiéter dans l'interprétation de ses résultats. Il ne semble pas admettre la possibilité de positions intermédiaires, qui pourraient cependant expliquer facilement ses résultats obtenus aux stades jeunes avant l'apparition de la ligne primitive. Des stades plus avancés n'ont pas été pris en considération non plus, pour éviter l'objection que la distance entre le centre du blastoderme et plus en particulier de l'area pellucida d'une part, et l'extrémité antérieure de la ligne primitive déjà plus longue d'autre part ne soit devenue trop petite et ne mène à des conclusions erronées.

Après section, les fragments sont mis en incubation sur le milieu préconisé par Spratt (1947) dans des salières dont le couvercle transparent est luté à la paraffine. Ceci rend possible de suivre à intervalles réguliers l'évolution des fragments et plus en particulier leurs mouvements morphogénétiques.

Après 24 heures de culture les fragments sont fixés dans le liquide de Zenker et les coupes sériées sont colorées selon la méthode de Unna–Brachet. Dans le cadre des recherches présentes nous avons réalisé 128 cultures et nous en avons examiné 49 sur coupes sériées.

OBSERVATIONS PERSONNELLES

Évolution des fragments antérieurs

Les fragments antérieurs évoluent comme Spratt (1942) le décrit. Dans notre série expérimentale, 13 des 19 fragments antérieurs examinés sur coupes et qui ne sont composés au moment de la section que d'un feuillet ectophyllique et d'un feuillet entophyllique, forment du tissu nerveux ou neuroïde. Le plus souvent, les plissements morphogénétiques qui accompagnent normalement la neurulation ne se produisent pas, mais trois fois nous avons vu des indications de structures rappelant un cerveau. Ce problème mérite certainement d'être approfondi. Ce qui nous intéresse toutefois plus particulièrement ici, c'est l'absence totale de formations révélatrices de l'existence d'un nœud de Hensen. Tout comme Spratt, nous n'avons jamais trouvé de chorde à l'examen microscopique. Mais, ce qui nous semble presque plus significatif encore, c'est que nous n'avons jamais observé de mouvement réel au niveau du bord de section, pendant les premières heures après la section: ni encoche formée par la ligne primitive qui éventuellement continuerait sa formation au delà de la section, ni formation, quelques heures plus tard, de la 'queue' tellement typique d'un nœud de Hensen en régression. Comment Spratt ose conclure en 1946, lorsqu'il réfère à ses expériences de 1942 qui sont presque identiques aux nôtres et à des expériences comparables réalisées à des stades plus jeunes ou plus avancés, dont nous avons fait la critique, que 'all of the notochord regions are shown by this method to be located far anterior to the anterior end of the short-beginning streak', nous échappe. Nous ne sommes pas les seuls d'ailleurs: Fraser (1954), un élève de Spratt, déduit de ces mêmes expériences qu'un mouvement vers l'avant du matériel chordal se produit pendant la formation de la ligne primitive, mais sous l'ectoblaste. Prudemment il s'abstient d'émettre une opinion sur la signification de l'extrémité antérieure de l'ébauche de la ligne primitive.

Évolution des fragments postérieurs

L'évolution des fragments postérieurs plaide encore plus fortement en faveur de la conception qui localise le futur nœud de Hensen dans la jeune ligne primitive. L'observation de ces fragments sous la loupe binoculaire au cours de l'incubation est très suggestive à ce propos (Fig. 1). En effet, au cours des six premières heures, la ligne primitive s'allonge considérablement. Le fragment qui la contient en subit des changements de forme très importants, qui peuvent mener jusqu'à un rétrécissement au milieu, dû évidemment à un manque de matériel disponible. Après 24 heures de culture le raccourcissement de la ligne primitive s'est opéré plus ou moins normalement. Au microscope nous reconnaissons du tissu nerveux d'aspect spinal, de la chorde, et des lames latérales. Ceci suggère qu'un processus au moins analogue à la régression du nœud de Hensen s'est déroulé.

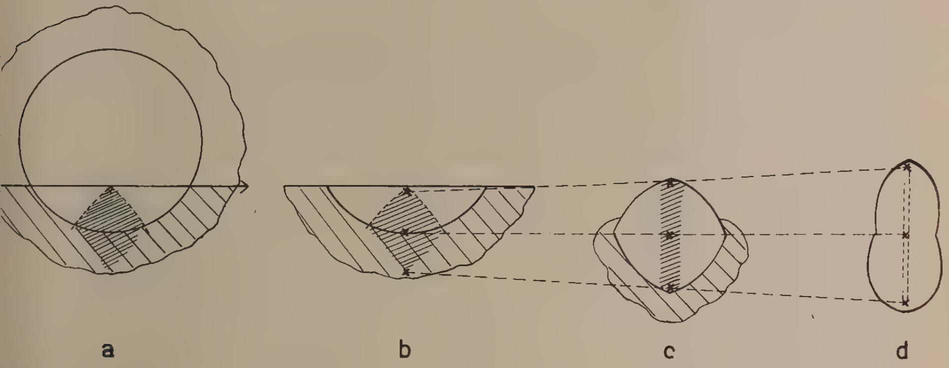

FIG. 1. Les dessins résument schématiquement le développement de fragments postérieurs de blastoderme de Poulet isolés en culture *in vitro*. *a*, blastoderme entier, montrant l'ébauche de la ligne primitive, de forme triangulaire dans l'area pellucida, moins nettement délimitée dans l'area opaca. La ligne de section est indiquée par une flèche. *b*, fragment postérieur isolé, l'emplacement des marques est indiqué par des croix. *c*, fragment postérieur après 3 heures de culture. Élongation, dans les deux sens, de l'axe de la ligne primitive. Agrandissement de l'area pellucida surtout au détriment de l'area opaca. *d*, après 6–8 heures de culture l'élongation a pratiquement atteint son maximum. Le sillon de la ligne primitive est apparu. Un rétrécissement de l'area pellucida est visible au niveau du milieu de la ligne primitive. L'area opaca, aux contours trop irréguliers et inconstants, n'est plus dessinée.

Des marques de charbon ont été appliquées quinze fois sur les fragments postérieurs en trois points: aux extrémités antérieure et postérieure et au milieu de la jeune ligne primitive. Ce dernier point correspond à peu près à la limite entre area pellucida et area opaca. De l'évolution de ces marques il nous a été possible de déduire que, du moins dans les circonstances de l'expérience, l'élongation de la ligne primitive se fait dans les deux sens de son axe: vers l'avant et vers l'arrière, et ceci à des vitesses et avec des extensions sensiblement égales. Dans des blastodermes isolés et entiers ce mouvement vers l'avant ne peut être noté que rarement, et cela seulement dans les blastodermes qui se développent le mieux. Le plus souvent, la distance entre les marques antérieure et moyenne ne varie pas ou guère, seul le mouvement vers l'arrière se retrouve régulièrement. Dans ces cas l'inhibition de l'extension antérieure de la ligne primitive doit être mise en rapport avec l'absence d'épibolie globale du blastoderme, qui se traduit par une diminution plutôt qu'un accroissement de la surface de ce dernier. La mise en place du système nerveux est donc pratiquement arrêtée, et on conçoit aisément que ce tissu massif situé — d'après tous les auteurs — en avant du matériel chordal, gêne considérablement l'extension vers l'avant de la ligne primitive.

Que ce raisonnement n'est pas purement inductif, trouve une confirmation dans le mode de développement de quelques lignes primitives qui se forment dans des blastodermes intacts, à partir du stade étudié. Nous avons vu trois fois sur ces blastodermes que la ligne primitive se termine vers l'avant par un pertuis qui se prolonge dans un petit canal. Des observations analogues ont été faites par Jacobson (1938), ce qui nous a convaincu de rapporter les nôtres malgré leur nombre restreint. Cette formation d'un 'canal blastoporal' illustre d'une façon très nette la tendance à la migration vers l'avant du matériel nodal. Elle permet d'affirmer que ce qui ralentit le plus l'extension du matériel nodal en culture est l'absence d'une épibolie normale du blastoderme, puisque ce matériel paraît beaucoup moins gêné par les conditions de culture, dès qu'il trouve un chemin profond. Chez les Amniotes dont l'épibolie est moins poussée que chez les Oiseaux, on trouve régulièrement un blastopore plus ou moins tubulaire. C'est ainsi que l'épibolie est lente chez les Reptiles, où l'on sait que la gastrulation s'effectue entièrement à travers un canal blastoporal. Les Mammifères dont l'épibolie est limitée, possèdent un canal de Lieberkühn à l'extrémité antérieure de leur ligne primitive, et présentent de ce point de vue un aspect intermédiaire entre les Oiseaux et les Reptiles.

L'extension de la ligne primitive vers l'arrière est plus généralement considérée comme une élongation. Nous pouvons soutenir cette opinion par nos observations. Lorsqu'on élimine entièrement l'area opaca d'un blastoderme au stade où la ligne primitive est à peine ébauchée, on voit une protrusion en direction caudale constituer la partie postérieure de la ligne primitive qui se forme. Nous avons observé une seule fois une petite queue qui constituait la partie postérieure de la ligne primitive en formation, et qui s'est éparpillée après quelques heures, ce qui permet de supposer qu'elle était essentiellement composée de mésoblaste non enveloppé par un autre feuillet.

DISCUSSION ET CONCLUSIONS

En définitive nous croyons donc avoir apporté des arguments qui confirment l'importance, au cours de la gastrulation de l'œuf des Oiseaux, de la région de transition entre l'area pellucida et l'area opaca et cela surtout à l'endroit où va se former la ligne primitive. Le fait de considérer cette région comme le centre d'une extension réelle vers l'avant et vers l'arrière de la ligne primitive, permet de comprendre les opinions radicalement opposées de Pasteels et de Spratt, parce qu'il permet d'illustrer l'influence que peuvent avoir les circonstances de la culture *in vitro*. Nous avons vu en effet que dans les conditions où Spratt a effectué ses marques au charbon, c'est-à-dire sur des blastodermes entiers, l'absence d'épibolie inhibe dans la ligne primitive pratiquement tout mouvement dirigé vers l'avant. Nous avons au contraire pu mettre en évidence cette tendance en permettant à la jeune ligne primitive de s'étendre, libérée de l'entrave qu'est pour elle la partie antérieure du blastoderme *in vitro*. Nos résultats donnent dès lors raison à Pasteels dans la controverse qui nous avait intrigué et nous font admettre que l'extrémité antérieure de la très jeune ligne primitive contient déjà le matériel du futur nœud de Hensen. Par une technique semblable à celle que Spratt utilise nous arrivons à confirmer les vues de Pasteels, basées sur des colorations vitales *in ovo*.

Il est d'autre part remarquable que nos expériences et celles de nos prédécesseurs nous amènent à situer le 'centre de l'organisateur' là où au cours de la prégastrulation l'endophylle se constitue en premier lieu et où son contact avec l'ectophylle est par conséquent le plus précoce et plus intime, ceci sans présumer de la façon exacte selon laquelle se forme l'endophylle. Ce point situé au milieu de la ligne primitive est le même vers lequel convergeront plus tard le nœud de Hensen et le nœud postérieur, et où se situera le bourgeon caudal, comme nous l'avons exposé dans un article récent (Vakaet, 1960).

SUMMARY

Isolated anterior and posterior fragments of very young blastoderms at the primitive-streak stage, transected in front of the streak, have been cultured for 24 hours. The posterior fragments were marked by carbon particles. Our results stress the importance, during bird gastrulation, of the transition region between the area opaca and the area pellucida, especially at the site of formation of the primitive streak. Considering this region as an extension centre, forward and backward, of the primitive streak enables us to understand the radically opposed opinions of Pasteels and Spratt by furnishing illustrations of the influence of *in vitro* culture circumstances. We have seen, indeed, that under Spratt's marking conditions on whole blastoderms, absence of epiboly practically inhibits all forward movement in the blastoderm. On the contrary, however, this last tendency is seen very clearly in our experiments where, by releasing it from the hindrance formed by the anterior part of the blastoderm *in vitro*, we enable the

streak to stretch forward. Our results, therefore, confirm the findings of Pasteels with regard to his controversy with Spratt and show that the anterior end of the very young primitive streak already contains the material of the future Hensen's node. Using Spratt's technique we arrive at a confirmation of Pasteels's opinions based on *in ovo* staining.

It seems worth while to note that our experiments and those of our predecessors tend to localize the 'organization centre' in the region where earlier the formation of the endophyll started and as a consequence its contact with the ectophyll is the most precocious and the most intimate. This is stated without any implication as to the exact mode of formation of the inner germ layer. On the other hand, this point in the midst of the primitive streak is the point of convergence of Hensen's node and the posterior node, and the place where the tail-bud will arise, as we have previously shown (Vakaet, 1960).

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Factors Responsible for the Abnormal Development of Embryos Obtained by Nuclear Transplantation in *Xenopus laevis*

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INTRODUCTION

In *Xenopus* the embryos derived from nuclear transplantation often develop abnormally. These abnormalities must be due to the limited potentiality for development of either the donor nucleus or the egg cytoplasm; this limited potentiality will in turn be due to technical damage during transplantation or to the innate condition of the nucleus or cytoplasm before the experiment. The extent to which these technical and innate factors are responsible for abnormalities of transplant-embryo development has been analysed by considering the effect of each factor in turn. Nuclei from early donor stages have been used, since these nuclei are believed to be undifferentiated (see p. 338) and therefore to have the innate capacity for entirely normal development. The effects of other factors have been investigated by experiments in which each factor is varied in different ways. Any correlation between variations in one factor and the resulting proportion of abnormal transplant-embryos is then recorded. In this way the effect of each factor on the development of transplant-embryos can be analysed.

These experiments show how nuclei can be transplanted with least damage, and whether small inconsistencies in carrying out the technique can bring about abnormalities of development. It is thus made clear to what extent abnormal transplant-embryos may be considered to represent innate qualities of donor nuclei.

TECHNIQUE

As the method of nuclear transplantation for *Xenopus laevis* is to be described elsewhere (Elsdale, Gurdon, & Fischberg, 1960), it will be only very briefly outlined here. Cells are dissected as a tissue from the donor embryo, disaggregated chemically, and then kept in saline solution until required. Unfertilized recipient eggs are irradiated with ultraviolet light to kill the egg pronucleus. A donor cell is sucked up into a pipette which is sufficiently narrow

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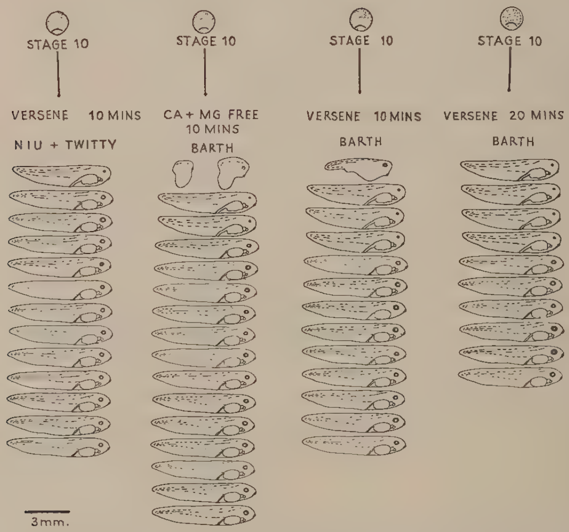
to disrupt the cell wall and leave the nucleus intact, protected by some cytoplasm. The nucleus with its cytoplasm is then injected into the recipient egg. This sequence of events justifies the distinction made below between the treatment of donor nuclei and that of recipient eggs.

DONOR NUCLEI

The disaggregation and storage of donor cells

Donor cells are prepared by placing the isolated tissue (dissected from the embryo with needles) in a disaggregating solution for between 10 and 20 minutes. During this time many cells become free, and they are then placed in a full-strength saline solution until required for transplantation. The disaggregating solutions are made from standard saline solution either by omitting the calcium and magnesium salts, or by replacing these salts with 5×10^{-4} M versene. Only the saline solutions of Niu and Twitty (Flickinger, 1949), and Barth (Barth & Barth, 1959) have been tested. Briggs & King (1953) have used *Rana* for nuclear transplantation and have found that Niu and Twitty saline causes fewer



Text-fig. 1. Donor cells from 4 early gastrulae were prepared in different ways and 36 transplantations made from each donor embryo; only transplant-embryos that cleaved regularly are shown. The disaggregating solution was the same as the solution in which cells were kept for transplantation, but with either Ca and Mg salts omitted, or with these replaced by 5×10^{-4} M versene. In this and all following text-figures, nearly normal tadpoles can be distinguished from normal ones by their small eyes and heart oedema.

abnormalities of development than many organic and inorganic solutions which they also tested. Since then Moore (unpublished), also using *Rana*, has found that fewer abnormalities resulted from the use of Barth's *X*-solution, which mainly differs from Niu and Twitty saline in containing $1\frac{1}{2}$ times as much NaCl.

In Text-fig. 1 the three columns on the left compare the effects of different disaggregating solutions on the same kind of donor cells. In this case there is very little difference between calcium- and magnesium-free Barth, Barth-versene, and Niu and Twitty-versene; in other experiments there is usually no difference between Barth- and Niu and Twitty-versene solutions, though more severe abnormalities are found after using Ca- and Mg-free Barth solution which has a weaker disaggregating effect than versene. Thus inadequate disaggregation seems to result in nuclear damage. This may be due to the fact that cells insufficiently disaggregated by chemical means tend to be separated mechanically when being transferred to the standard saline solution; this may result in damage to the cell wall, which then fails to protect the nucleus from the saline solution. King & Briggs (1955) have found that donor cells are less damaged if disaggregated chemically than if dissected apart with needles. The conclusion from this experiment is that the nuclei of donor cells are more likely to be damaged after insufficient dissociation than by any direct effects of versene.

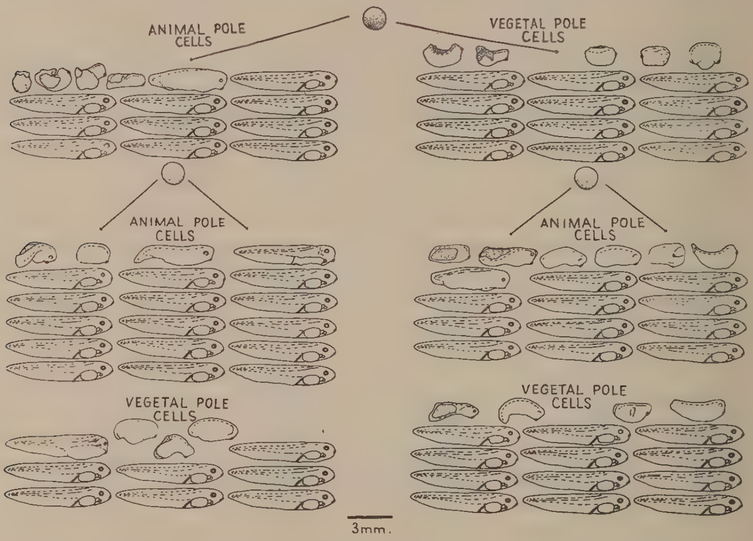
To test whether disaggregation by even the *best* method was nevertheless harmful, donor cells were left for varying periods of time in Barth-versene. Cells that were kept for longer times in versene were left for correspondingly shorter times in the standard solution; thus the total time for which cells were in any saline solution was constant. The two columns on the right of Text-fig. 1 show that there is no pronounced difference in results if the donor cells are left for twice as long as is necessary in versene solution. These tests have been carried out on blastula cells the nuclei of which are relatively well protected by cytoplasm. Endoderm nuclei from hatched tadpoles have also been transplanted after exposure to versene solution for varying periods of time. Donor cells disaggregated for 20 minutes and 35 minutes gave equally good results, which were better than those obtained from cells disaggregated for only 10 minutes. It is apparent that versene in the strength used does not damage nuclei of intact cells even if they are exposed to it for double the normal time.

These results mean that care should be taken to disaggregate donor cells thoroughly before transplanting their nuclei, and that exposure of cells to versene for longer than the usual 10–15 minutes is not harmful.

The treatment of donor cells in the injection pipette

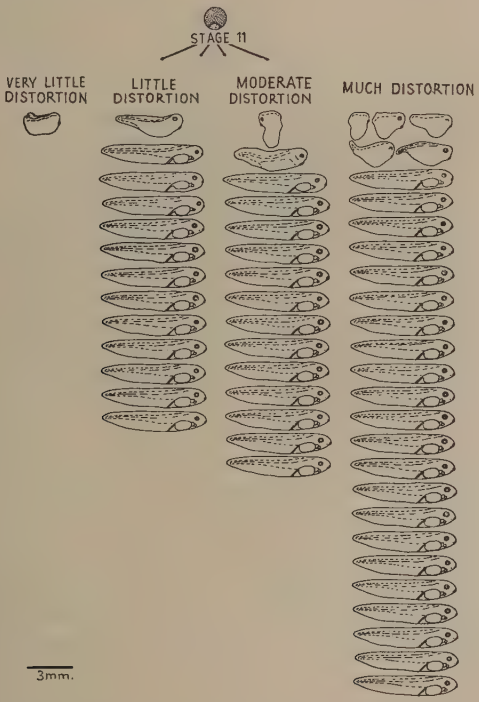
At this stage in the technique there are two ways in which the donor nuclei may be damaged. They may either be physically broken or distorted when being sucked up into the micropipette, or they may be chemically harmed by exposure to the saline solution. Each kind of damage is likely to be increased the more the donor cell is distorted when sucked into the pipette. Two kinds of experiment have been carried out to investigate this source of damage.

The first experiment involved transplanting animal and vegetal cells from blastula donor embryos. Cells from these regions differ considerably in size, though their nuclei are of approximately the same volume. Both kinds of cells were distorted to the same extent, by sucking them into pipettes of different sizes; thus animal cell nuclei were protected by a thinner layer of cytoplasm, and so were more likely to be exposed to the solution; animal cell nuclei were also pushed more out of shape than the vegetal nuclei. If transplanted nuclei are affected by either of these factors, embryos derived from animal cell nuclei should develop more abnormally than those derived from vegetal nuclei.



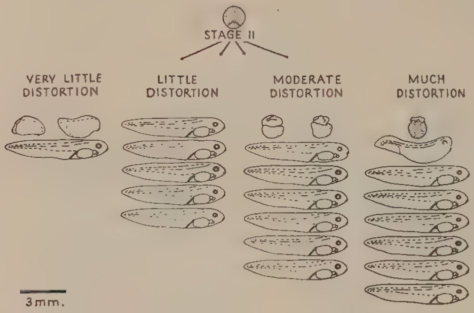
TEXT-FIG. 2. Transplantations were made from undifferentiated nuclei of small animal and large vegetal cells of a blastula donor. A blastula transplant-embryo from each of these groups served as a donor for two serial clones, in each of which transplantations from animal and vegetal cells are shown separately.

In Text-fig. 2 this test has, in effect, been carried out 3 times; the fact that two of the blastula donor embryos happen to have been derived from the first by nuclear transplantation does not affect the present conclusions. Though some abnormal embryos are present in each transplantation group, it can be seen that there is no consistent difference in the proportion of abnormalities derived from animal or vegetal donor nuclei. This shows that the smaller amount of cytoplasmic protection possessed by animal hemisphere nuclei does not expose them to technical damage. The chief importance of this result concerns the interpretation of experiments in which nuclei are taken from much later donor stages. The endoderm cells of hatched prefeeding tadpoles, though slightly smaller than blastula ectoderm cells, have a similar ratio of nucleus to cell volume. This means that differences in the result of transplanting endoderm nuclei from early and late stages cannot be due to the smaller size of the cells rendering them more susceptible to technical damage.



TEXT-FIG. 3A. Cells from an early gastrula donor, disaggregated by Barth-versene, have been transplanted into recipient eggs of one frog. The distortion of donor cells on being sucked into the pipette was varied, but in all other respects the technique and materials were the same for the four groups, in each of which 36 transplantations were made; only transplant-embryos which cleaved regularly are shown.

In the second experiment different sized pipettes were used for donor cells of the same size and kind as each other; the donor cells were therefore distorted by the pipette to different degrees. Four categories of distortion have been recognized (Text-fig. 3). The ratio between the diameter and length of the cell inside the pipette was about 2:3 for 'very little distorted' cells, about 1:2 for 'little distorted' cells, about 1:3 for 'moderate distortion', and 1:4 or 5 for 'much distortion'. In cells which were very little distorted the cell wall was often not broken and no development took place; in much distorted cells the cytoplasm was much dispersed so that nuclei may have come into direct contact with the saline solution.



TEXT-FIG. 3B. Similar to Text-fig. 3A in all respects except that eggs were obtained from a different recipient frog and that only 12 transplantations were made in each group.

Text-fig. 3 shows two experiments of this kind. Within each, exactly the same number of transplantations were carried out for each group. These diagrams, however, show only those embryos that became regular late blastulae. Since the cause of abnormal cleavage after transplantation is very uncertain, eggs which cleaved irregularly have been omitted from the diagrams; but they became, in fact, more frequent as the distortion of the donor cell decreased. The most obvious result, equally clear in each experiment, is that the proportion of transplanted eggs which cleave normally varies directly according to the amount of distortion of the donor cell. The abnormalities in each group should be considered as a proportion of the number of embryos shown. Rather surprisingly, embryos derived from 'very little distorted' donor cells develop very abnormally. Among the other three groups the proportion of abnormal embryos and the severity of abnormalities slightly increases with increase in distortion.

These experiments clearly show that to obtain the best results, donor cells should be distorted to a small or moderate degree, forming in the pipette a cylinder of length 2–3 times its diameter. If cells are distorted too little, the cell wall is often not broken properly and this results in much abnormal cleavage, though for no obvious reason the few regular late blastulae that occur also develop very abnormally. If donor cells are distorted too much, the abnormalities increase a little; this is presumably because the nuclei are exposed to the saline solution, as strong distortion causes the cell cytoplasm to be mixed with the saline solution.

When interpreting nuclear transplantation experiments it is very important to remember that the amount of distortion of the donor cell is by no means the only factor that decides what proportion of transplanted eggs will cleave normally. In practice it is very difficult to distort each donor cell to exactly the same extent, so that up to 20 per cent. of variation in the percentage of normal cleavage will probably not convey information about the quality of donor nuclei. On the other hand, nuclei from late donor stages give *conspicuously* fewer normal late blastulae than those from early stages, even though both kinds of cells are distorted to a very similar extent (Gurdon, 1960*b*).

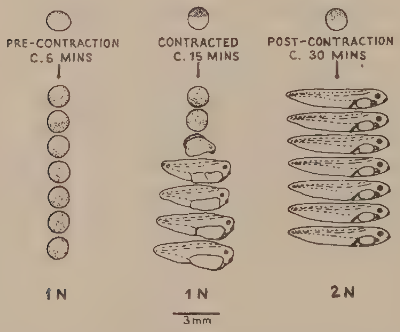
RECIPIENT EGGS

Technical treatment of recipient eggs

The treatment to which recipient eggs are subjected during transplantation consists of: ultraviolet (UV) irradiation, penetration by a micropipette, and the deposition near their centres of a nucleus, some donor cell cytoplasm, and a small amount of saline solution. In addition, the eggs remain unsupported by a fluid medium during these operations. These factors will be discussed in turn.

The main purpose of UV treatment is to kill the egg nucleus, but it also renders the egg more easily penetrable to a micropipette by dissolving some of the jelly and weakening the vitelline membrane. The UV treatment of uncleaved *Xenopus* eggs is the subject of another publication (Gurdon, 1960*a*). Several experiments are described, none of which give any reason to believe that the UV irradiation of the animal hemisphere damages the egg cytoplasm. The clearest evidence was given by an experiment in which fertilized eggs were irradiated for different lengths of time. As the UV kills the egg pronucleus, fertilized irradiated eggs develop as androgenetic haploids, which are never entirely normal. However, in this experiment no change in the proportion of abnormal embryos was obtained, even though the UV dose was considerably more than that normally used for transplantation experiments.

The donor cell cytoplasm, which is never more than one 10,000th of the egg by volume, has been found to have no effect on development when injected (without a nucleus) into a fertilized egg. The remaining three factors (the penetration by a pipette, injection of saline solution, and lack of support by fluid) were first tested with fertilized eggs. Eggs were treated in three ways: (*a*) UV-irradiated only, (*b*) UV-irradiated and penetrated by a pipette, but with no fluid injected, and (*c*) UV-irradiated, penetrated by a pipette, and a little Barth saline solution (but no nucleus) injected as for transplantation experiments. In each case the eggs were left unsupported by fluid for the usual amount of time, i.e. 5–10 minutes. In this experiment again all embryos were haploid, and therefore none were entirely normal. The results varied considerably from the eggs of one frog to those of another; for this reason a typical experiment cannot be shown. There is a general tendency for most abnormalities to be derived from eggs which were irradiated and injected with saline solution (*c*), and for eggs which had been irradiated only (*a*) to develop most normally. These differences may be due to some extent to haploid embryos being able to recover less well from injection with solution than diploids. By whatever means haploid *Xenopus* are obtained, some develop more abnormally than others (Gurdon, 1960*a*). It is unlikely that the poor developmental capacity of haploids can account for all the differences between eggs irradiated only (*a*), and eggs irradiated and injected (*c*). Since the difference between the three kinds of treatment is not apparent with some eggs but pronounced with others, it seems clear that the ability to withstand the transplantation treatment depends on egg quality. This matter is discussed further in the next section. As it is unfortunately not possible to obtain diploid development from *fertilized* eggs treated for transplantation, further tests were carried out on *un*fertilized eggs with transplanted nuclei. The amount of saline solution injected into the eggs was varied from about the same volume as the donor cell to 2 or 3 times this amount. This had no effect on the proportion of abnormalities among the results. The time for which eggs remained unsupported by fluid could be increased to at least 20 minutes (which is double the usual time) without having any effects, so long as the surface of the eggs did not become dry.



TEXT-FIG. 4. Fertilized eggs, UV-irradiated at different intervals after laying, were all treated as for nuclear transplantation; they were penetrated with a pipette and injected with saline solution (but no nucleus). All embryos were haploid in the left and central groups, but diploid in the right-hand group.

The eggs of *Xenopus* often undergo a contraction of the area of animal pole pigment and at the same time the consistency of the eggs seems to become stiffer (Gurdon, 1960*a*). The duration and time of onset of this contracted state varies from the eggs of one ovulation to those of another; in each egg it usually starts 10–20 minutes after laying and lasts for about 10 minutes. Text-fig. 4 shows the result of irradiating fertilized eggs and injecting them with saline solution (but no nucleus) at different times after laying. The eggs treated before contraction have all become arrested as late blastulae, but those in the contracted state have developed as well as haploids usually do. In those treated after the contracted state, the egg nucleus has had time to sink below the surface and so is protected from the UV; these have therefore become diploid and all are normal. It seems that there is associated with this contraction an increased resistance to technical interference such as penetration by a pipette (Text-fig. 4). It is not clear whether this contraction of *Xenopus* eggs is comparable to the activation of *Rana pipiens* eggs (Briggs & King, 1953). The present author has also found (Gurdon, 1960*a*) that experimental manipulations, such as enucleation with a needle, result in developmental abnormalities if carried out on eggs immediately after they have been laid, but can be much less harmful if carried out about 15 minutes after laying. This applies to both fertilized and unfertilized eggs. For this reason recipient eggs are used for nuclear transplantation about 15 minutes after laying, when they are usually in the contracted state.

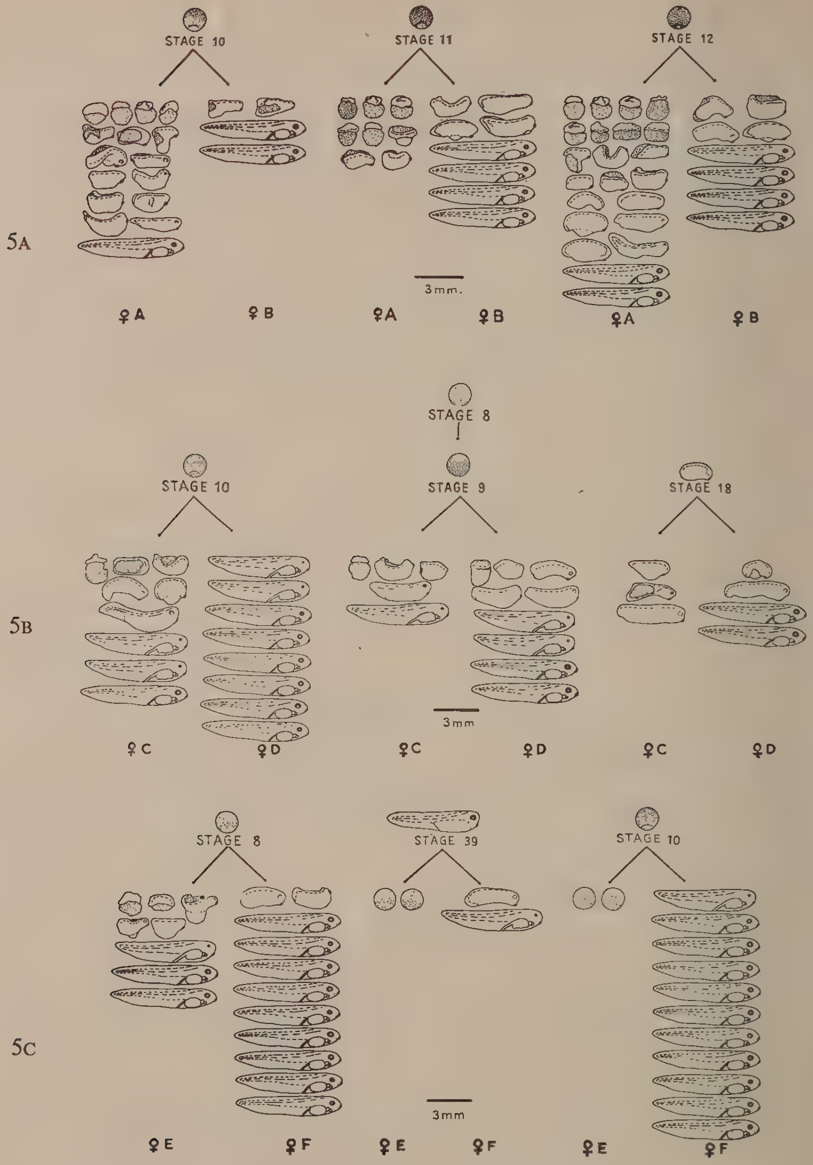
The above tests have shown that there is no reason to believe that the usual treatment of good recipient eggs, during transplantation, in any way reduces their capacity to develop normally. Though this seems to apply to the eggs of most ovulations there may be some exceptions, since the quality of eggs varies (see next section).

Innate variation in the quality of recipient eggs

There seems to be a considerable discrepancy in the results obtained from recipient eggs of different female frogs, in spite of using the same donor cells. This was tested by transplanting nuclei from a single donor embryo into groups of eggs from two frogs laying at the same time. The results of these experiments are shown in Text-fig. 5. In these diagrams there are two groups of embryos under each donor. The left-hand group comes from the eggs of one frog, and the right-hand group from those of another. In each case a very marked difference between the two groups of embryos can be seen, which must show a difference in egg quality since there was no other variable in these experiments. The poor eggs differ from the good ones not only in the greater proportion of developmental abnormalities, but also in the smaller number of regular late blastulae obtained (especially Text-fig. 5C). This difference in the quality of eggs persists whatever donor stage is used (compare Text-fig. 5 B, C). The eggs of one female are usually of the same quality throughout one ovulation, though there is a tendency for the last eggs laid to give worse results, presumably because some eggs are immature. One female may lay good eggs at one ovulation but bad ones at the next, and it has not been found that certain females can be relied upon to give good or bad eggs every time they lay.

Many attempts have been made to improve the quality of eggs, as for instance by varying the diet, but so far none have been successful. Frogs recently imported from the wild do not always give much better eggs than ones bred in our laboratory. Eggs are obtained from each female by injection of gonadotropic hormone at 2- to 3-month intervals.

This variation in the results obtained from the eggs of different females is rather surprising in view of the fact that naturally fertilized eggs from hormone-injected frogs nearly always develop very well; usually over 90 per cent. of such fertilized eggs develop into normally feeding tadpoles. It is possible that the



Text-fig. 5. These experiments, in which there was no variation in technique, show how transplant-embryo development may vary according to the quality of recipient eggs. In each figure the eggs of two recipient frogs have been used and nuclei from each donor embryo have been transplanted into the eggs of both frogs. The figures are set out according to the order in which the transplantations were made, i.e. nuclei from the donor on the left were transplanted first and from the donor on the right of each figure last: only transplant-embryos which cleaved regularly are shown.

Text-fig. 5A. About 30 transplantations were made from early, middle, and late gastrula donors into the injection of a nucleus brings about development in some eggs which, owing to poor quality, would not have been fertilized or which would not have developed normally if they had been fertilized. It is found on the average that a higher percentage of eggs cleave regularly after transplantation than after fertilization. On the other hand, it may be that eggs can develop quite normally when fertilized, but are nevertheless very variable in their ability to withstand experimental manipulations. In either case the cause of variation is egg quality. This observation does not apply only to nuclear transplantation experiments, since it is known that embryos derived from the eggs of different frogs are also very variable in their ability to heal after tissue grafting.

No way has yet been found of distinguishing recently laid eggs which will develop well after nuclear transplantation from those which will not. It was mentioned above that recipient eggs are not usually used for transplantation until they have undergone certain changes recognized by contraction of the area of animal hemisphere pigment. While the selection of contracted eggs gives fewer abnormalities (Text-fig. 4), it is not found that all contracted eggs give equally good results. It is quite possible for the eggs of one frog to cleave well, and even to gastrulate well, but later to suffer from many more abnormalities than those of another frog. The quality of eggs is therefore judged entirely by the proportion of normal tadpoles obtained from them. This means it is necessary to start and end all experiments with a control series—that is, with donor nuclei the developmental potentialities of which are already known. Only if these control transplantations develop well can attention be paid to the results obtained from other donor nuclei.

DISCUSSION AND CONCLUSIONS

There are four main factors which determine the way in which embryos from transplanted nuclei develop: these are the technical treatment and innate qualities of donor nuclei and recipient eggs. The majority of the experiments carried out have consisted of nuclear transplantations, since this is the most direct way of investigating these factors.

The technical treatment of donor nuclei can be carried out in such a way that it is harmless. To achieve this cells should be thoroughly disaggregated by exposure to versene for 10–20 minutes according to the stage of the donor embryo. When sucked up into a pipette donor cells should be distorted so that their length is 2–3 times their diameter. In practice, small variations within this

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eggs of each frog. The eggs of both frogs gave abnormal transplant-embryos but the ratio of abnormal to normal embryos was appreciably greater for female *A* than for female *B*.

TEXT-FIG. 5B. About 25 transplantations were made from a gastrula, serial blastula, and neural folds, donor to the eggs of each frog. Female *C* gave consistently worse results than female *D*.

TEXT-FIG. 5C. About 30 transplantations were made from a blastula, hatched tadpole, and gastrula donor into the eggs of each frog. Female *F* gave exceptionally good and consistent results, while the eggs of female *E* gave very poor results, and appear to have deteriorated in quality during the experiment.

range of cell distortion are unavoidable; these do not affect the further development of blastula transplant-embryos, but may account for up to 20 per cent. of variation in the number of total transplantations which form regular blastulae.

The effects of the technical treatment and innate qualities of recipient eggs will be discussed together since it is difficult to distinguish between them. The innate quality of eggs is often responsible for variation in transplant-embryo development. It was shown that undifferentiated donor nuclei, when transplanted under the same conditions, can give rise to very different results according to the female's eggs into which they were transplanted. These different results may either be due to variation in the innate developmental capacity of eggs, or to variation in their capacity to withstand, or recover from, the experimental manipulations to which they are subjected. In either case the cause of variable results is egg quality. A considerable reduction in abnormalities was obtained by using recipient eggs in the contracted state, but in spite of this the eggs of some frogs gave much better results than those of others. Unfortunately no way has yet been found of improving egg quality, thereby eradicating this source of abnormalities, or of recognizing the quality of eggs other than by the development to which they give rise. This makes it necessary to carry out a control series of transplantations using early-stage donor nuclei, the developmental potentialities of which are already known. If the recipient eggs are of good quality these control transplantations will develop as expected, and attention can be paid to the results of the experiment; if the controls develop very abnormally, the whole experiment must be discarded. Thus technical treatment or innate quality of recipient eggs will not affect the conclusions if these are restricted to experiments in which egg quality proved to be good.

The innate qualities of donor nuclei were kept constant; this was done by using nuclei from early donor stages which are believed to be undifferentiated as shown in the experiment described below. One of the main uses of nuclear transplantation is to test the innate qualities of different kinds of nuclei; experiments of this kind, which are described elsewhere (Gurdon, 1960*b*), can only be done when the effects of other factors have been excluded.

The effects of all four factors which influence transplant-embryo development have now been considered; a standard technique has been developed by which experiments can be carried out and analysed in such a way that the only variable factor is the innate quality of donor nuclei. This is demonstrated by the following experiment in which early gastrula endoderm nuclei were used as donors. Fourteen nuclei were transplanted; 3 did not cleave but the remaining 11 all cleaved normally. These 11 regular late blastulae all formed completely normal feeding tadpoles, which would almost certainly have metamorphosed as usual if they had not been fixed. These results give good reasons for believing that the endoderm cell nuclei of blastulae and early gastrulae are all totipotent, or capable of giving normal transplant-embryo development. It shows that if all conditions are perfect (most especially the quality of recipient eggs), every undifferentiated nucleus which initiates cleavage will bring about normal development. This experiment is atypical, since in most cases a few abnormal embryos occur in series which otherwise consist of normal tadpoles. However, its importance is not decreased by its uniqueness, since the failure of other experiments to give such clear results seems to be due to the quality of recipient eggs.

SUMMARY

1. An analysis has been made of factors which can cause abnormal development of embryos derived from transplanted nuclei in *Xenopus laevis*. A distinction is made between innate and technical factors, and between those which affect donor nuclei and recipient eggs.

2. The innate developmental potentialities of endoderm donor nuclei do not appear to be limited, at least up to the early gastrula stage. Abnormalities known to be due to differentiated donor nuclei from later donor stages are described elsewhere. The technical treatment of donor nuclei can be carried out so as not to damage them, even if this is purposely made more severe than usual.

3. The innate capacity for development of recipient eggs was found to vary considerably from one spawning to another; this variation seems to be due to an inability of the eggs of any one frog to withstand technical treatment which may be shown to be harmless to the eggs of other frogs.

4. As the quality of recipient eggs can only be judged from results, experiments using *Xenopus* eggs should be accompanied by control transplantations, using donor nuclei the developmental capacity of which is already known. With these control transplantations for comparison, the technique is suitable for testing unknown developmental potentialities of different kinds of donor nuclei or recipient eggs.

RÉSUMÉ

Les facteurs responsables du développement anormal des embryons de Xenopus laevis *obtenus par transplantation nucléaire*

1. Il a été procédé à une analyse des facteurs qui peuvent provoquer le développement anormal des embryons obtenus chez *Xenopus laevis* par transplantation de noyaux. On fait une distinction entre facteurs innés et techniques, ainsi qu'entre ceux qui affectent les noyaux du donneur et les œufs qui les reçoivent.

2. Les potentialités innées de développement des noyaux provenant de l'endoderme du donneur ne paraissent pas être limitées, du moins pas jusqu'au stade de gastrula jeune. Les anomalies dont il est connu qu'elles sont dues à des noyaux différenciés de donneurs parvenus à des stades plus avancés sont décrites ailleurs. Le traitement technique des noyaux donneurs peut être réalisé de façon à ne pas les léser, même si ce traitement est rendu à dessein plus sévère que d'habitude.

3. Il a été constaté que la capacité innée de développement des œufs pris

comme récepteurs varie considérablement d'une ponte à une autre; cette variation paraît tenir à l'incapacité des œufs de telle grenouille à résister à un traitement technique qui s'est avéré inoffensif pour les œufs d'autres grenouilles.

4. Comme la qualité des œufs récepteurs ne peut être appréciée que d'après les résultats, les expériences utilisant les œufs de Xénope devraient être accompagnées de transplantations contrôles, utilisant des noyaux dont la capacité de développement est déjà connue. A condition de recourir pour comparaison à ces transplantations contrôles, la technique convient pour mettre à l'épreuve les potentialités inconnues de développement que possèdent divers types de noyaux du donneur ou d'œufs récepteurs.

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Some Degenerative Phenomena in *Drosophila* Ovaries

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WITH FOUR PLATES

INTRODUCTION

THERE are several reasons why it is of interest to examine the ultra-structure of degenerating cells. In the first place, processes of cellular degeneration may be involved in normal developmental processes. In the formation of the insect egg, for instance, a great deal of the ooplasm is derived from the substance of the nurse cells, which degenerate and eventually disappear as the oocyte reaches its final stage of growth. Again, the follicle cells, after secreting the chorion, degenerate and eventually disappear. As will be demonstrated in this paper, degenerating cytoplasm often exhibits some rather well-organized and elaborate structures which might have been taken, if one did not know of the cells in which they were found, to be organelles concerned with synthetic activities. A study of such materials suggests the dangers of too easy interpretation of some of the appearances seen in healthy cells.

The degeneration of the nurse cells, and the passage of material from them into the oocyte, in the normal development of the wild type *Drosophila* ovary will be considered in another paper. Three types of degenerative phenomena will be discussed here: (1) the degeneration of the follicle cells; (2) changes taking place in unfertilized eggs subsequent to laying; and (3) degenerative changes in the nurse cells of flies homozygous for the female-sterile gene *deep-orange*. Females homozygous for this gene can produce fertilizable eggs which die at an early stage of development unless fertilized by sperm carrying the normal allelomorph of the gene (Counce, 1956). In the ovaries of such females, however, many egg-chambers degenerate and fail to produce fully mature oocytes. The appearances to be described here have been seen in such degenerating egg-chambers: the conditions in the more normal eggs which may be fertilized and begin development will be described in a later article.

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MATERIALS AND METHODS

The ovaries were dissected out of females (at age 2 days) in *Drosophila* saline. Fixation was by 1 per cent. osmium tetroxide, dissolved either in buffered saline, or better, in buffered sucrose. Embedding in methacrylate was carried out in the normal way, and sectioning was done with a thermal advance microtome. The sections, mounted on Formvar grids, were examined with an electron microscope (Siemens Elmiskop I).

The degeneration of the follicle cells

Each egg-chamber of a *Drosophila* ovary is clothed with a follicular epithelium, the cells of which are at first all of a roughly cuboidal shape. When the oocyte begins to enlarge, at about stage 7, the follicle cells lying against it become taller and more cylindrical. From approximately the same stage onwards the vitelline membrane begins to form between them and the oocyte; as was shown in a previous paper (Okada & Waddington, 1959) the formation of the first 'vitelline bodies' is accompanied by the appearance of droplets or vacuoles in the oocyte plasm, and the material for the vitelline membrane is probably produced by the oocyte rather than by the follicle cells. However, the formation of the vitelline membrane is closely followed in time (or accompanied by) the development of the chorion, and this is certainly produced by the follicle cells, being separated from the oocyte by the already-present vitelline membrane. After the completion of the chorion, the follicle cells above it shrink and eventually disappear.

At an early stage in the formation of the vitelline membrane, the cytoplasm of the follicle cells has a relatively homogeneous structure apart from the occurrence of mitochondria. There are few indications of an ergastoplasm composed of double membranes (Plate 1, fig. 1). As the formation of the chorion gets under way, double membrane structures become more obvious. This is quite in accordance with what one observes in other differentiating *Drosophila* cells, in which ergastoplasm makes an appearance just when the cell begins to lay down its characteristic product (Waddington & Perry, 1960) The point that is particularly interesting here is that the double-membranes go on becoming more and more obvious and massive even when the cells are rapidly shrinking, immediately prior to their final disappearance; that is to say, at a time when it seems likely that the secretion of the chorion has been completed. A photo of a follicle cell at an early stage in this process is given in fig. 2 of Plate 1; the specimen has probably been somewhat over-extracted during the histological preparation, but this serves only to show up more clearly the strongly developed ergastoplasmic system. An extremely striking system of concentric double-membranes from a similar or even older cell was illustrated by Okada & Waddington (1959, fig. 11).

Changes in unfertilized eggs

The ooplasm of the mature eggs is a comparatively well-organized structure, with an outer layer of finely granular cytoplasm containing many mitochondria, and with many yolk particles, lipid particles, and granular particles of lesser or greater electron density in the more central regions. A full description has been given by Okada & Waddington (1959). In eggs which are not fertilized, one finds that after a few hours there has been a considerable rearrangement of these various constituents, of such a kind that similar particles tend to come together. The electron-light granular particles, which have been referred to as beta particles, are probably more liquid than the rest of the egg. They run together into large masses around which the mitochondria become grouped (Plate 2, fig. 4). The granular ground substance of the cytoplasm also tends to become separated into two fractions, one of a denser and one of a lighter consistency. In a late stage of degeneration, these darker areas may sometimes show an arrangement of the particles into a periodic system of parallel membranes (Plate 2, fig. 5).

Degeneration in deep-orange

In *deep-orange* egg-chambers the anatomical appearances of degeneration are very variable. In some cases even the polarity of the egg-chambers is reversed, so that the oocyte lies at the wrong end of it. Degeneration may begin at various stages in oocyte development, either before or after yolk formation.

In oocytes which have reached an advanced level of differentiation before degeneration overtakes them, the appearances may be rather similar to those seen in unfertilized normal eggs, in that there is a considerable segregation of the ground substance into areas of different electron density (Plate 4, fig. 11). There is also a marked development of double laminar structures which often form sets of concentric rings. In other cases the cytoplasm becomes extremely vacuolated. Some of these vacuoles probably represent badly fixed lipid particles, but it seems unlikely that all of them can be accounted for in this way, and some of them are lined by well-defined membranous structures (cf. Plate 4, fig. 10). There are also patches of a peculiar 'fuzzy' appearance, round which there are often one or more double profiles (Plate 4, fig. 12). These could be considered as a new type of particle, never found in healthy cytoplasm. They probably represent, however, the degenerative derivatives of one of the types of granule characteristic of the oocyte, possibly the beta granules.

The 'annulated laminar stacks', described as normal constituents of the egg cytoplasm, can also be seen undergoing degeneration. This involves the splitting of each lamina into two membranes, between which vacuolar swellings appear (Plate 1, fig. 3). It was pointed out in the previous paper that these laminae normally have a structure which closely resembles that of the nuclear envelope, in which the presence of annular pores is obvious but the existence of two membranes is not very evident. The phenomena seen during degeneration make it plausible to suppose that the laminae are in fact always composed of two closely-adherent membranes even when this is not obvious.

In the *deep-orange* nurse cells affected by premature degeneration, the cytoplasm usually presents rather a homogeneous appearance. Some membrane-lined vesicles were found (Plate 4, fig. 10), and so were persistent mitochondria; the distribution of the latter is, however, rather peculiar even in chambers in which a fertilizable egg is being developed, and they will be considered more fully in a later paper.

The most striking feature in these nurse cells is the appearance of large masses of contorted porous membranes, which must represent the persistent remains of the nuclear envelopes. In healthy nurse cells at their fullest extent, these envelopes are very large and complexly folded (Plate 3, fig. 6). With progressive degeneration in *deep-orange* ovaries, the more electron-dense material inside the nucleus (nucleolar material) runs together into large more or less compact masses, while the nuclear envelope becomes fragmented (Plate 3, figs. 7, 8). The pieces of broken-up envelope rather frequently lie nearly parallel to each other, so that some regions of the complex look rather like the annulated laminar stacks (Plate 4, fig. 10). However, the whole mass of membrane is much larger and less well arranged than those stacks (Plate 3, fig. 9).

DISCUSSION

One point of interest in the degenerative phenomena described here is the appearance of quite elaborate structural organization in cytoplasm which is near the point of death. The segregation of components in the unfertilized eggs is, perhaps, not very surprising, but at least it indicates that inhomogeneities are not always evidence for the occurrence of vital metabolic processes. The lamellar structures found in these degenerating eggs are also a warning that caution is necessary in interpreting the functional significance of ergastoplasm-like formations, and this is reinforced by the persistence and even strengthening of typical double-membrane systems in the cytoplasm of degenerating follicle cells. It has been found that in the developing eyes of *Drosophila*, ergastoplasm usually becomes well developed in cells of any given type just at the time when the characteristic cell-product (such as rhabdomere, cornea, pigment, &c.) is being rapidly produced; and in most of these types of cells the double-membranes later regress to some extent at least (Waddington & Perry, 1960). The phenomena described in the follicle cells show that this later regression is not an inevitable accompaniment of cessation of differentiation. It appears, however, as yet too early to attempt a full understanding of the relation between the appearance of ergastoplasm and the anabolic and katabolic processes of the cell; much more information, on a wider range of differentiating cells, as well as of adult cells engaged in secretion, will be necessary before we have a satisfactory basis for this. In the meantime it seems advisable to keep such appearances in mind when interpreting, for instance, the very remarkable structures described by Bellairs (1958) in yolk granules in the cells of chick embryos.

The complexes of fragmented nuclear envelopes seen in *deep-orange* ovaries are, in the first place, an indication that this structure has considerable solidity and resistance to autolysis. Perhaps the most interesting point about them is the frequent arrangement of the fragments into groups of parallel double-laminae. This could be most easily interpreted by attributing it to some relatively long-range forces, perhaps of the nature of van der Waals forces. It is noteworthy that the associations which arise in this way always involve intervals between contiguous structures which are considerably greater than the gap between the two members of the nuclear envelope, or that between the two membranes composing each lamina in the annulated laminar stacks. It seems probable that the forces holding the membranes together in paired structures, such as nuclear envelopes and the laminae in the stacks, are rather markedly different from those by which the parallel associations of fragments of nuclear envelope are brought about. One is reminded of the difference between the pairing of half-chromatids in mitotic prophase and metaphase, and the looser associations which sometimes occur in somatic pairing. The distinction between these two types of association is, however, probably not complete, since in degenerating laminar stacks the two members of a lamina may become more widely separated by the appearance of vacuoles between them, as in fig. 3 of Plate 1. The membranes composing the double structures of the ergastoplasm may also exhibit all degrees of separation, from an association almost as close as that seen in the nuclear envelope to the appearance of an extensive sac-like lumen between them. It is, however, surely significant that single and unpaired solid structures are so rarely seen in living systems. Most membranes turn out to be double-membranes, and when there are a number of pieces of such membrane, these are often found to be arranged roughly parallel to one another. The forces which bring about this arrangement must almost certainly be related to those involved in chromosome pairing and in the formation of the structures seen in unfertilized eggs.

SUMMARY

A description is given of degenerative phenomena in the *Drosophila* ovary. Three types of degeneration are dealt with: (1) degeneration of the follicle cells; (2) degeneration occurring in the unfertilized egg, a major feature of which is the segregation of the different constituents of the egg into comparatively large masses; and (3) degenerative phenomena in the nurse cells and oocytes of abnormal eggs produced in ovaries homozygous for *deep-orange*.

In all these instances it is noteworthy that a considerable degree of structural organization (formation of double membranes, or sets of parallel or concentric membranes, &c.) occurs in cytoplasm which is clearly degenerating. It is suggested that such organization does not always represent the formation of functioning organelles, but may be a mere consequence of the inherent physicochemical properties of the materials involved.

In degenerating *deep-orange* ovaries masses of fragmented porous membranes are found. These seem to be derived from the nuclear membranes of the nurse cells, which appear to persist even when the rest of the cell is rather completely destroyed. Fragments of nuclear envelopes tend to lie close together and parallel to one another, and it is suggested that this indicates that there is some force of attraction between them, which may be comparable to the forces involved in the formation of stacks of annulate lamellae, or of concentric or parallel double-membrane structures in ergastoplasm, and in chromosome pairing in healthy functional cells.

RÉSUMÉ

Sur quelques phénomènes de dégénérescence dans les ovaires de Drosophila

Une description est donnée des phénomènes de dégénérescence dans l'ovaire de *Drosophile*. On distingue trois types de dégénérescence: (1) la dégénérescence des cellules folliculaires, (2) la dégénérescence qui se produit dans l'œuf non fécondé dont un caractère principal est la ségrégation des différents constituants de l'œuf en masses relativement importantes, et (3) des phénomènes de dégénérescence dans les cellules nourricières et les ovocytes d'œufs anormaux formés dans les ovaires homozygotes pour le facteur *orange sombre*.

Dans tous les cas, il est remarquable de voir d'importants phénomènes d'organisation structurale (formation de doubles membranes, ou de séries de membranes parallèles ou concentriques, etc.) dans un cytoplasme qui est nettement en voie de dégénérescence. Il est suggéré qu'une telle organisation ne représente pas toujours la formation d'organites fonctionnels, mais qu'elle peut être une simple conséquence des propriétés physico-chimiques inhérentes au matériel considéré.

Dans les ovaires *orange sombre* en dégénérescence, on trouve des amas de membranes poreuses fragmentées. Ils semblent dériver des membranes nucléaires des cellules nourricières, qui paraissent persister même quand le reste de la cellule est presque complètement détruit. Des fragments de membranes nucléaires tendent à se rassembler parallèlement les uns aux autres, et l'on suppose que cela indique que des forces d'attraction les rapprochent.

Ces forces peuvent être comparées à celles qui interviennent dans la formation de piles de lamelles annulaires, ou de doubles membranes concentriques ou parallèles dans l'ergastoplasme, et aussi dans l'appariement des chromosomes des cellules saines et fonctionnelles.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Parts of two follicle cells, stage 8. The external membrane is visible at the top, and from it an intercellular boundary runs down to just below the middle, on the left. At the bottom is a small area of oocyte cytoplasm, with the vitelline membrane just beginning to appear between it and the follicle cells. The follicle cell nucleus, with nucleolus, is seen at the mid-region of the right-hand cell. The cytoplasm contains many mitochondria (less dense than the cytoplasm ground substance, which is here unduly granular), but few double-membranes, though some are visible at the top of the cell and just below the nucleus. ×24,000.

FIG. 2. Follicle cell cytoplasm, stage 12, towards the end of the formation of the chorion, which is seen at the bottom right. The section does not contain a follicle cell nucleus. Ergastoplasmic double lamellae very strongly developed. ×8,000 approx.

FIG. 3. Degenerating annulated laminar stack, in cytoplasm of a *deep-orange* egg. ×43,000.

PLATE 2

FIG. 4. Unfertilized egg, $1\frac{1}{2}$ hours after laying. The cytoplasm has segregated into large areas of different density, the light areas probably being formed from beta granules. Mitochondria (MIT) have accumulated along the margins between areas of different density, and lipid granules (represented by spaces) have also come together in groups. ×15,000.

FIG. 5. Unfertilized egg, $1\frac{1}{2}$ hours after laying. The cytoplasm has again segregated into areas of different density. A light area lies above, a dark one below, with mitochondria accumulated along the boundary. The cytoplasm of the dark area has formed a series of roughly parallel bands. ×24,000.

PLATE 3

FIG. 6. Part (about one-sixth or less) of the nucleus of a wild-type nurse cell, stage 11, to illustrate the great extent of the highly folded nuclear envelope. The internal contents of the nucleus contains much scattered dark 'nucleolar' material; the cytoplasm, generally paler in tone, carries a few mitochondria and lipid granules. ×10,000.

FIG. 7. Degenerating *deep-orange* nurse cells, to show partial break-up of the nuclear envelope. The darker patches are presumably 'nucleolar' material. ×3,000.

FIG. 8. Persisting nuclear envelope in a *deep-orange* egg-chamber. Note the parallelism of several fragments, e.g. at the right near the top. ×15,000.

FIG. 9. Extensively fragmented nuclear envelope material in a *deep-orange* nurse cell. Contrast with the background has already been partly lost. Note the many parallel arrangements. ×36,000.

PLATE 4

FIG. 10. Structures in a degenerating *deep-orange* nurse cell. The annulated membranes above (NNM) are presumably derived from the nuclear membrane. Below are vesicles (VES) with associated concentric membranes, and some well-preserved mitochondria (MIT). × 43,000.

FIG. 11. A degenerating *deep-orange* ovarian egg. The vitelline membrane (VM) is well formed, and the egg must have been fully mature before beginning to degenerate. The contents have segregated, as in unfertilized normal eggs, and a number of concentric double-membrane rings have appeared. × 18,000.

FIG. 12. A 'fluffy body' with associated double membrane structure, in the cytoplasm of a degenerating *deep-orange* egg. × 33,000.

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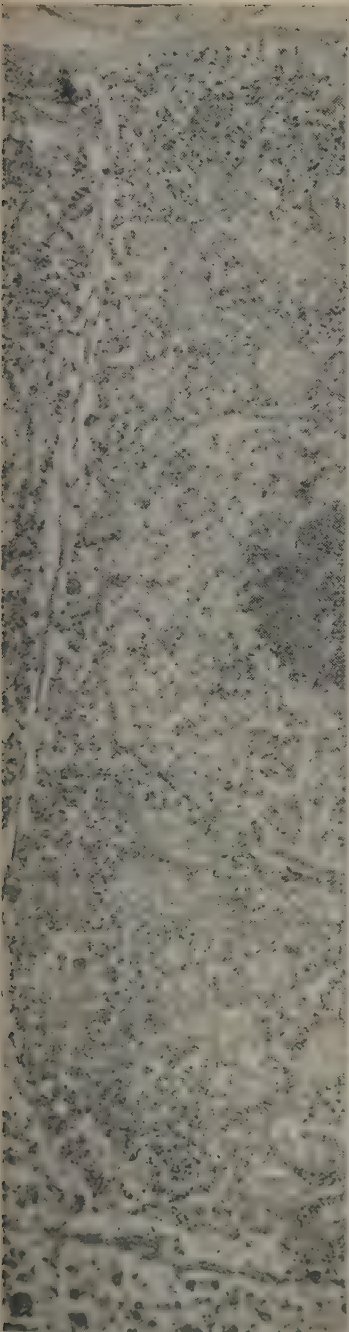

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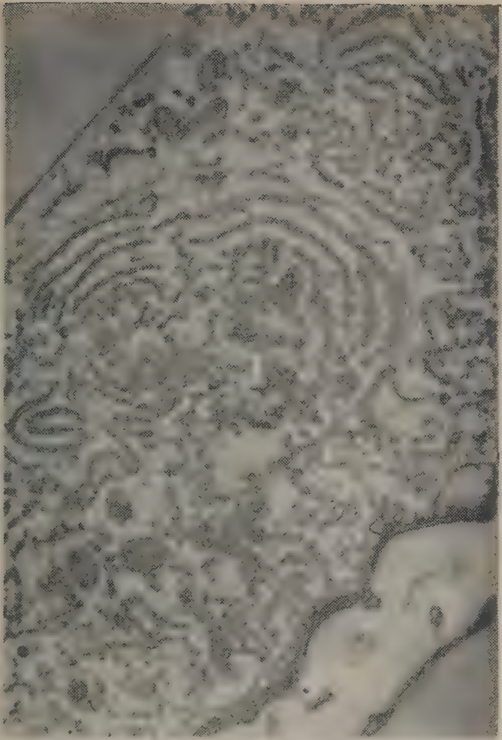

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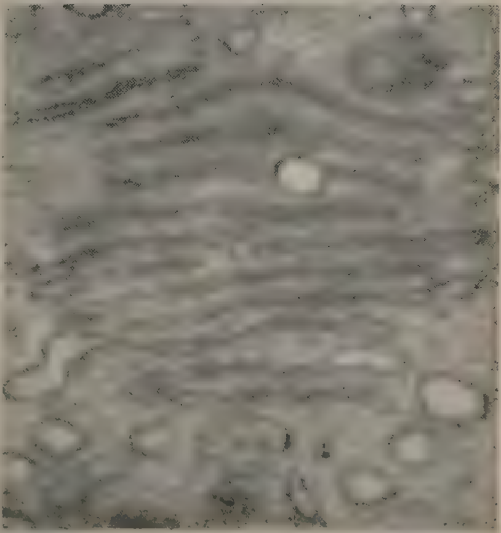

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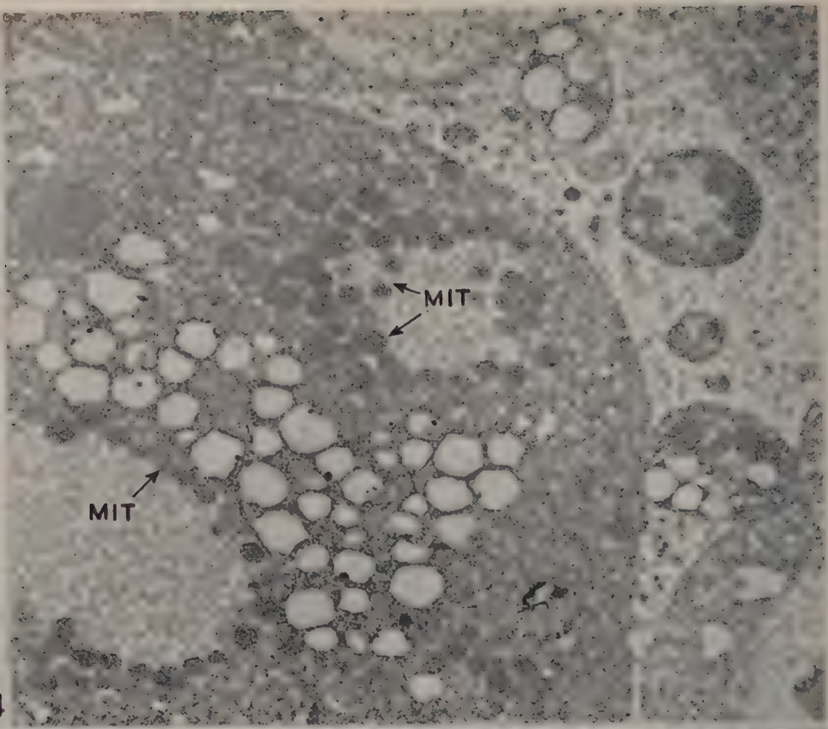
Plate 1



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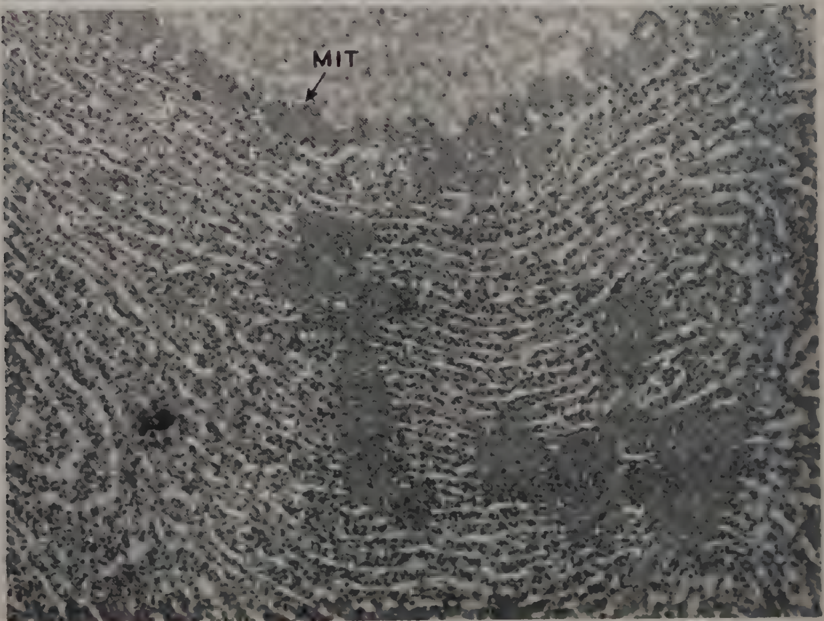

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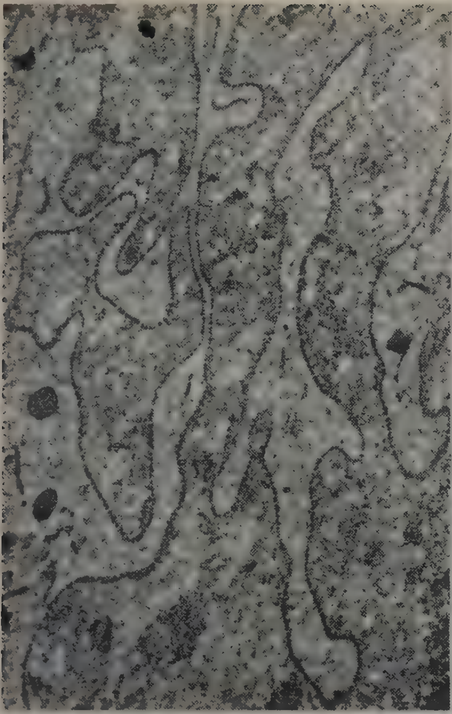
Plate 2



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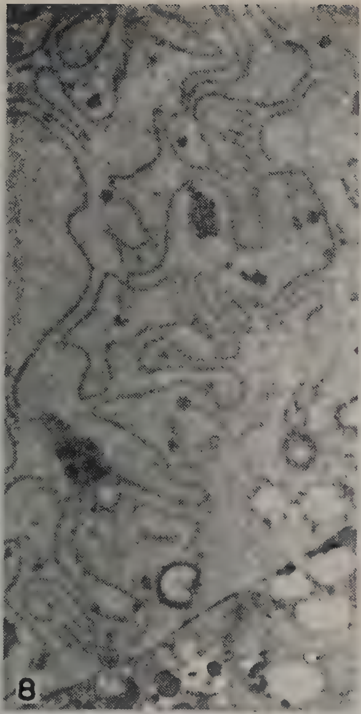

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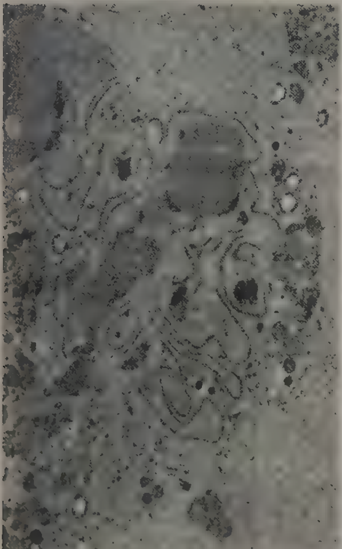

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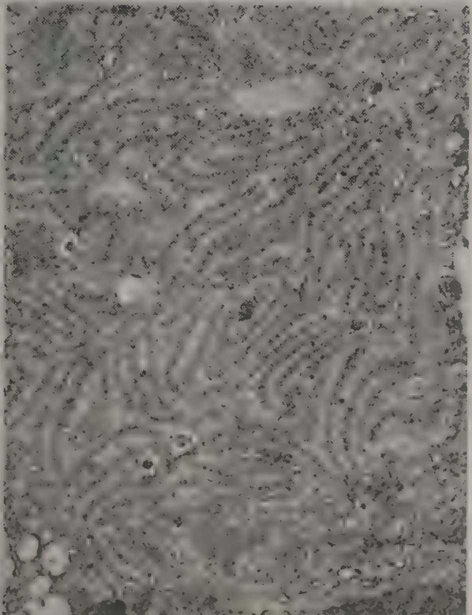

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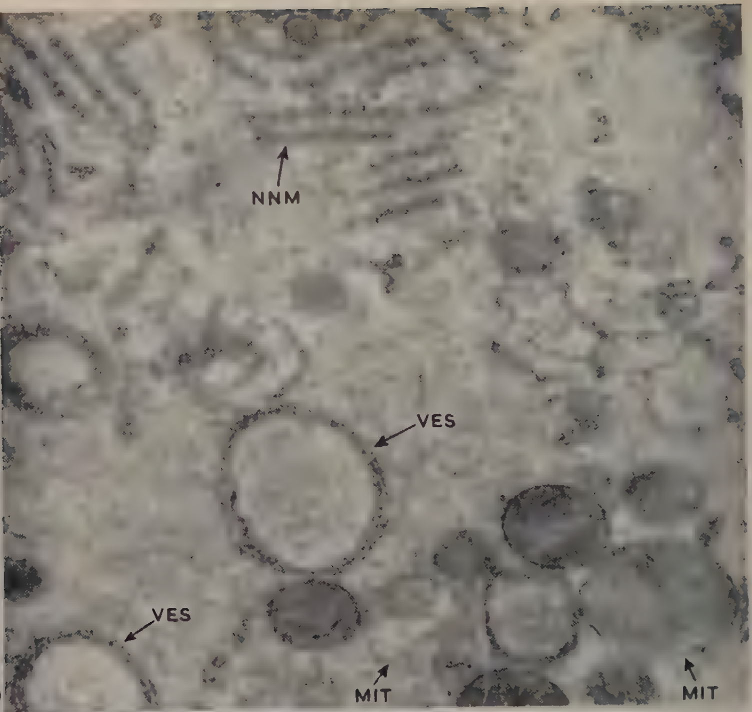
Plate 3



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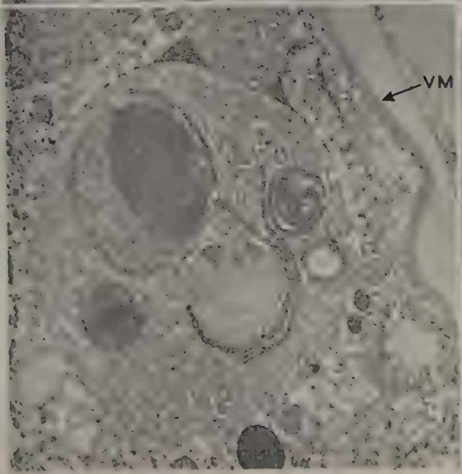

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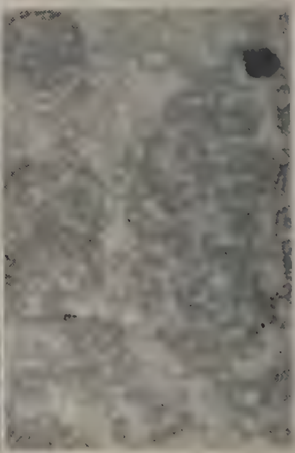

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C. H. WADDINGTON *and* E. OKADA

Plate 4

Experimental Analysis of the Eye Morphogenesis in Mammals

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WITH FOUR PLATES

OWING to the great difficulties of experimental interference with the embryogenesis of mammals, the morphogenetic potentialities of the mammalian eye remain almost unstudied up to the present. The elucidation of whether the developing mammalian eye obeys the same rules that have been established for the lower vertebrates, or whether its development proceeds in another way, is interesting in many respects. It might allow us to answer a number of questions, e.g. how stable are the morphogenetic interactions within the vertebrates which result in the formation of homologous organs? How do the morphogenetic potentials of the parts of an organ alter in the course of evolution? Light might also be thrown on the development or loss of regenerative capacity in various forms. Lastly, we may find out to what extent the experiments on lower vertebrates, easily carried out on a large scale, are of use for the understanding of those processes which cause regularly recurring inborn malformations in man.

The external eye-layer, i.e. the pigmented epithelium of the retina which plays the role of a light-reflecting screen in the functioning eye, is a very interesting structure morphogenetically. In such forms as newts it has the capacity of metaplastic transformation during the whole life of the animal, for it can regenerate other parts of the eye such as retina, iris, and lens (Wachs, 1920; Stone, 1950 *a*, *b*; Reyer, 1956; Stone & Steinitz, 1957; Hasegawa, 1958); other animals may possess this ability during larval life (Anura: Sato, 1953; Lopashov, 1955; Stroeva, 1956*a*) or only during the early embryonic stages, this capacity being lost when the external layer becomes pigmented (Acipenserid fishes—Dabaghian, 1959), or even prior to its pigmentation (birds—Alexander, 1937; Dorris, 1938; Gayer, 1942; Reinbold, 1958).

After the work of Dragomirov (1936, 1938, 1939), repeated by Detwiler & van Dyke (1953, 1954), it became known that the neural retina could be induced in the presumptive epithelium of the eye rudiment in newts and frogs by contact with the auditory vesicle or with the ectoderm. Later on Stone (1950*b*) showed

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that it was only the pigmented epithelium which was the source of the regenerated retina. Finally, Lopashov (1945, 1946, 1948, 1951, 1960), when explanting eye rudiments and transplanting them into different areas of the embryonic body, was able to show that the originally homogeneous eye rudiment could either differentiate into pigmented epithelium or into retina, according to the developmental conditions of its cells. Thus both the external (pigmented epithelium) and the internal (retina) layers of the eye represent an example of divergent differentiation from a rudiment originally homogeneous in its morphogenetic properties. One of the initial factors determining the differentiation of the amphibian pigmented epithelium is the mesenchyme enveloping the wall of the eye rudiment. When coming into contact with this latter, its cells become flattened and arranged in one layer, and an accumulation of pigment granules begins to take place in them. A sharp increase of the external layer pigmentation takes place when blood circulation is established. On the other hand, under conditions of condensation of the eye-rudiment cells, and in the absence of both the mesenchyme and the wash blood, they give rise to neural retina. The same was shown to hold true for the embryos of Acipenserid fishes (Dabaghian, 1958, 1959).

No special investigations have been made on the differentiation mechanism of the retina and the pigmented epithelium in birds. A number of data show, however, that in birds the differentiation of the eye rudiment obeys the same rules as in amphibians. Pieces of the external eye-rudiment layer of a chick embryo, as well as those of the internal one (up to 76–80 hours of incubation) differentiate in tissue culture into the typical pigmented epithelium when grown as a thin layer on the surface of the plasma clot; on the other hand, in a liquid medium, in which the external layer forms a concentrated mass, it sometimes forms retina (Dorris, 1938). Indications of the important role of the mesenchyme in the differentiation of pigmented epithelium in birds are to be found in the work of Gayer (1942). When transplanting eye rudiments with surrounding tissues (stage of 1–24 somites) taken from normal chick embryos and from the *Creeper* line with hereditary colobomas, a normal pigmented epithelium develops in the well-developed scleral and choroid coats of both lines. Defects in mesenchyme coats are accompanied by the development of the retina in the external eye layer.

The present investigation aims at elucidating the question of whether or not the external layer of the mammalian eye rudiment is able to undergo transformation into retina and, if it is, within what range of embryonic developmental stages it may occur; and whether or not the differentiation of the external layer depends on its contact with the mesenchyme. The answer to these questions was obtained by comparing the development of eye rudiments surrounded by the mesenchyme in the anterior chamber of the eyes of adult animals with those lacking surrounding mesenchyme (for preliminary report, see Stroeva, 1956*b*). Unfortunately, the converse situation, i.e. the placing of the presumptive retina into an environment of mesenchyme in order to elucidate whether the internal layer of the eye rudiment is able to differentiate into the pigmented epithelium, cannot be realized in this type of the experiment.

Having mainly in view the investigation of morphogenetic potentialities of the pigmented epithelium, the present experiments suggested some ideas with respect to the general conditions of eye morphogenesis, such as the role of the lens in the formation of the eye-cup and the role of extension of the retina for its vitality, growth, and differentiation.

MATERIAL AND METHODS

The experiments to be reported here were carried out on grey rats of a line cultivated for 10 years in our laboratory. Eye rudiments were taken at four stages of embryonic development as follows: eye vesicle (11·5 days, Plate 1, fig. A); eye-cup prior to the onset of pigmentation in its external layer (12·5 days, Plate 1, figs. B, C); eye-cup at the onset of pigmentation in the external layer and closure of the choroid fissure margins (13·5 days, Plate 1, figs. D, E); and eye-cup at the time of complete closure of the choroid fissure (14·5 days, Plate 1, figs. F, G). These rudiments were implanted into the anterior chamber of an adult eye. The transplantations were carried out under aseptic conditions through a linear cornea incision near the limbus by means of a fine glass pipette, the diameter of which somewhat exceeded that of the implant. Prior to implantation, eye rudiments with surrounding tissues were removed from the embryos in Tyrode solution at 37·5° C. or at room temperature, and were either immediately implanted into the anterior chamber of the adult eye or exposed to enzyme treatment with trypsin by the method of Moscona (1952) in order to release them from their mesenchyme coats. To this end, eye rudiments were washed four times in Tyrode solution lacking Ca and Mg ions (Moscona solution) and put into 3 per cent. trypsin prepared in Moscona solution. After 5 minutes in trypsin (at 37·5° C.), eye rudiments were again washed 4 times in Moscona solution and put into Tyrode solution, in which the mesenchyme was stripped from them with the help of slight touches with a small knife; this often caused the lens rudiment to fall out. Trypsin as well as Tyrode and Moscona solutions were sterilized by filtration through a Seitz filter. In some early experiments the mesenchyme was separated surgically without enzymic treatment. Neither method ensured complete elimination of the mesenchyme in all cases.

In order to elucidate whether trypsin injured the implant as a whole, or specifically affected the transformation of the pigmented epithelium, 36 implants belonging to the series in which eye rudiments were implanted with their surrounding tissues were also treated with trypsin but without the subsequent separation of the mesenchyme. Histological analysis showed the implants to become looser under the effect of trypsin, but in their further development they did not differ from the implants unexposed to enzyme treatment. This allows us to assume that trypsin does not specifically affect morphogenetic potentialities of the eye, and therefore the implants which received enzymic treatment are not separated in the presentation of our results.

In a preliminary series (A; 16 rudiments) carried out in order to elucidate whether the eye rudiments are able to undergo development and normal organogenesis in the anterior chamber of the adult eye, it was shown that eye rudiments of the first two ages, when taken together with the surrounding mesenchyme and lens ectoderm, do develop organotypically. A total of 500 transplantations of eye rudiments at all four ages were carried out in the main series. They can be divided into two types of experiments: implantation of eye rudiments without surrounding tissues (B) and with them (C). The principal time of fixation was the 6-8th day of cultivation for the first two stages, and the 4th day for the two later ones. All of them were histologically analysed (sections 8 μ thick; staining by Heidenhain's Azan method), and 254 of them are included in the description of the results obtained. As a supplement to series B a small series of experiments on the implantation of the inner layer, with the lens placode taken at the stage of 12·5 days (7 implants) and fixed 11, 28, and 31 days after implantation, was performed.

Simultaneously with the experiments, serial preparations of the eyes of normal animals belonging to the same rat line from the age of 12·5 days of embryonic development up to 15 days of postnatal life (174 preparations) were carried out. No deviations from the normal pigmented epithelium and retinal differentiation were found in any of these cases.

Material on eye rudiment implantation is supplemented by cases of external eye-layer transformation into retina in rat embryos as a result of X-ray and thermal shock treatment (Svetlov & Korsakova, 1954, 1960) on pregnant females (9–11 days of pregnancy; fixation on the 17th day of pregnancy) (section D). These cases were obtained in the Laboratory of Embryology, Institute of Experimental Medicine (Leningrad), and kindly put at my disposal for histological analysis by Professor P. G. Svetlov.

RESULTS

A. *Preliminary series*

Eye-cup formation in the implants taken from the eye vesicle stage, and pigmentation of the external layer in the implants of both stages, at first proceed at the same rate as in the controls; after 3–4 days differentiation of the pigmented epithelium and the retina prove to be quite distinct. The vitreous chamber is always lacking and the retina closely adjoins the lens, which is often vacuolated. Later on the growth of the eye in the implant and the differentiation of the retina lag behind the control, and necrosis appears in the retina after 11 days of cultivation. However, in the implants, which are cultivated for 11, 15, or 17 days, one may find the retina with all its typical layers but lacking the outgrowths of the visual cells. Outside the eye, in the depth of the mesenchyme, cartilage and, in the case of eye vesicle implantation, large brain vesicles, are differentiated. Proceeding from these data, the time of fixation was chosen for the main series.

B. *Implantation of eye rudiments without the surrounding tissues*

1. *Eye rudiments at the age of 11·5 and 12·5 days*

Results of eye-rudiment cultivation at the first two stages are analysed together because of their similarity. Implants lacking the surrounding mesenchyme undergo transformation into retina (21 cases, Plate 1, fig. H) which, in the absence of the lens, loses the shape of the cup and becomes arranged in rosettes attached either to the cornea or to the iris, or to both. Its growth and differentiation are retarded, and necrosis is often to be seen in its centre. In most implants small areas of the pigmented epithelium are to be found. Of 119 implants studied (Table 1), 66 had small areas of pigmented epithelium which were accompanied by the adjoining mesenchyme (Plate 1, fig. K). In 29 implants areas of the pigmented epithelium arose in the absence of the embryonic mesenchyme, but 26 of them closely adjoined the iris of the host (Plate 1, fig. I). The latter almost always thickens at the site of its contact with the implant and surrounds it with mesenchyme, which can hardly be distinguished from that brought with the implant. To be exact, it should be noted that the part of the implant adjoining the iris of the host does not always differentiate into the layer of pigmented epithelium. Such differentiation most likely arises only when it is the presumptive pigmented epithelium of the implant which adjoins the iris, but not when it is the presumptive retina. Thus, in the absence of mesenchyme, the entire eye rudiment develops into retina; the differentiation of the pigmented epithelium is in most cases associated with contact of the implant areas with mesenchymal tissues.

2. *Internal layer with the lens placode at the age of 12·5 days*

The lens in the implants undergoes differentiation. The retina closes around the lens, or it lies in the form of an irregular cup (Plate 1, fig. I). In 5 cases a cavity developed between the retina and the lens. The retina differentiates into layers, and in the two specimens fixed latest the ganglions and the internal retinal, internal nuclear, external retinal, and external nuclear layers are represented though in a somewhat loosened state. No outgrowths of visual cells are formed. Differentiation of the iris and ciliary processes is observed in the regions of the inner layer adjoining the lens after 28 days of cultivation (an analysis of this phenomenon will be given in a separate communication). The occurrence of pigmented epithelium was not observed. Thus, when cultivated together with the lens, the retina arranges itself around it in the form of a cup; it does not form rosettes, and it undergoes further differentiation.

TABLE 1

Cultivation of eye rudiments taken at 11·5 and 12·5 days of development after the removal of mesenchyme coats

				Development of the expected type		*Deviations from the expected type*			
							Analysis of preceding column		
Stage of implant taken (days of embryonic development)	*Total number of transplantations*	*Number of implants lost or degenerated*	*Number of developing implants*	*Most of the implant is made up of retina, but small areas of pigmented epithelium underlain by mesenchyme have arisen*	*No mesenchyme, the entire implant is made up of retina*	*Mostly retina, but somewhere areas of pigmented epithelium have arisen without mesenchyme*	*Pigmented epithelium adjoining the host iris*	*Pigmented epithelium without connexion with the host iris*	*Retina surrounded by mesenchyme*
11·5	49	32	17	14	1	1	1	—	1
12·5	137	35	102	52	20	28	25	3	2
TOTAL	186	67	119 = 100%	66	21	29 = 24·37%	26 = 21·85%	3 = 2·53%	3 = 2·53%
				$\underbrace{\qquad\qquad}_{87\,=\,73{\cdot}1\%}$					

3. *Eye rudiments at the age of 13·5 and 14·5 days*

The experiments on the transplantations of eye rudiments without surrounding mesenchyme from older embryos (13·5 and 14·5 days) were unsuccessful. At the same time as becoming pigmented, the external layer of the eye rudiment gets thin and sticky. The eye, once it is freed from the mesenchyme, sticks to the walls or to the tip of the pipette during the process of implantation. This series of experiments allows us to state, however, that the pigmented epithelium acquires stickiness with the onset of its pigmentation; in normal development this stickiness apparently contributes to the realization of a closer contact between the pigmented epithelium and the mesenchyme.

C. *Implantation of eye rudiments with the surrounding tissues*

The present experiments differ from the preliminary series in their multiplicity and in the fact that they include transplantation of eye rudiments at all four stages. Eye rudiments here developed organotypically, as in the preliminary series. When extending the material, however, we obtained a considerable variability in the development of the external eye layer.

1. *Eye rudiments at the age of 11·5 and 12·5 days*

The formation of the eye rudiment itself proceeds in the same way in the implants obtained from embryos having an optic vesicle and from those having an eye-cup with an unpigmented external layer. In some of them the mesenchyme separates from the implant, and the external layer undergoes transformation into retina, the nerve fibres of which stretch over the surface of the implant since the polarity of this retina proved to be opposite to that of the retina differentiated from the internal layer (Plate 2, figs. A, B). Unlike series B 1, the external layer here preserves its individuality, which is demonstrative evidence of its ability to undergo transformation into retina. In other implants, as in the preliminary series, the whole layer forms a normal pigmented epithelium (Plate 2, fig. F). In another half of the cases, in spite of the surrounding mesenchyme, retinal areas are included in these or in other regions of the external layer. As can be seen from Table 2, retina can arise in any region of the external eye rudiment, but this often takes place on the pupillary margin (Plate 2, figs. C, E) or along the choroid fissure (Plate 2, fig. D). In some cases the pigmented epithelium layer differentiates on the external surface of such retinal areas which have arisen in the external layer (Plate 2, fig. E). In one case, a part of the external layer in a normally segregated eye rudiment was replaced by nerve fibres (Plate 2, figs. F, G). Concerning the normal pigmented epithelium, mesenchymal cells were, as a rule, oriented parallel to its surface, closely adjoining both the pigmented epithelium and each other. This rule is often broken along the retinal areas, but not in all cases.

When eye vesicles are implanted with the surrounding tissues only a part of each implant forms the eye-cup. Almost half of the anlage gives rise to a tissue

TABLE 2

Cultivation of eye rudiments taken at 11·5, 12·5, 13·5, and 14·5 days of embryonic development, together with the surrounding tissues

				Development of the expected type		*Deviations from the expected type*				
							Analysis of preceding column		*Pigmented epithelium*	
Stage of implant (days of embryonic development)	*Total number of transplantations*	*Number of implants lost or degenerated*	*Number of developing implants*	*Typical eye differentiation with the pigmented epithelium underlain by mesenchyme*	*Transformation of the pigmented epithelium into retina at the sites of mesenchyme separation*	*Retina in the external layer in the presence of mesenchyme*	*Retina formation on the pupillary margin or on that of the choroid fissure*	*Retina formation at other sites of the external layer*	*Without mesenchyme*	*Doubled*
11·5	28	12	16	5	1	10	6	4	—	—
12·5	131	59	72	20	15	37	25	12	—	—
TOTAL	159	71	88 = 100%	41 = 46·6%		47 = 53·4%	31 = 35·2%	16 — 18·2%	—	—
13·5	39	13	26 = 100%	13 16 = 61·5%	3	8 = 30·8%	8 = 30·8%	—	—	2 = 7·7%
14·5	13	11	20 = 100%	16 16 = 80%	—	2 = 10%	2 = 10%	—	—	2 = 10%

resembling the neural mesenchyme in appearance. It lies either outside the eye-cup or surrounds it, and the borders between this tissue and the external eye layer, which always acquire retinal structure at the site of its contact, get effaced (Plate 3, figs. A, B). In some rare cases migration of the neural mesenchyme from the external layer is also observed after implantation of a cup with unpigmented external layer. At the site where neural mesenchyme cells escape from the external layer the latter is, as a rule, also transformed into retina. The appearance of the migrating cells, the way in which they escape, and their mingling with the surrounding mesenchyme resemble the condition described by Bartelmez (1954) for the eye vesicle of human embryos.

2. *Eye rudiments at the age of 13·5 and 14·5 days*

When implanting eye rudiments of the two later stages, no migration of neural mesenchyme cells from the external layer was observed. In these cases attention is drawn first of all to the well-organized mesenchyme coats of the eye and to the regularly arranged pigmented epithelium (Plate 3, figs. D, E). In eye rudiments taken at the stage of 13·5 days retina also arises in the external layer (8 cases out of 26, Table 2), but always only on the pupillary margin or on the margin of the choroid fissure. Further, a doubling of the pigmented epithelium is observed near the pupillary margin and at the posterior pole of the eye. In 3 cases the mesenchyme separates from the external layer in various eye regions, and retinal areas of considerable thickness arise in these places (Plate 3, fig. C). This shows that the external layer completely preserves its ability to undergo transformation into retina even after the onset of pigmentation; however, in other regions than the pupillary margin or that of the choroid fissure this ability is displayed only in the absence of the mesenchyme. The external layer of the implants taken at the age of 14·5 days differentiates normally in most cases (16 cases, Table 2). Sometimes a doubling of the pigmented layer takes place (Plate 3, fig. F); in two cases small retinal formations arose along the pupillary margin (1 case), and along the margin of the choroid fissure (1 case; Plate 3, fig. F).

As to the general development in implants of this age, both the general growth of the rudiments and the proportional growth of their external and internal layers are disturbed, unlike that of the implants of the first two stages. This seems to be associated with the fact that the conditions of the anterior chamber become insufficient for the development of the eye rudiments at these later stages; thus, although the external layer differentiating into the normal pigmented epithelium does not suffer damage, retinal growth is strongly inhibited. As a result of this an abnormally large primary eye cavity is formed (Plate 3, fig. E). The lens is preserved in some cases only, while in most implants it gets partially or completely absorbed by the time of fixation. Both the sclera and cornea are differentiated in some cases; the margins of the ectoderm close above the latter, resembling eyelids (Plate 3, fig. D).

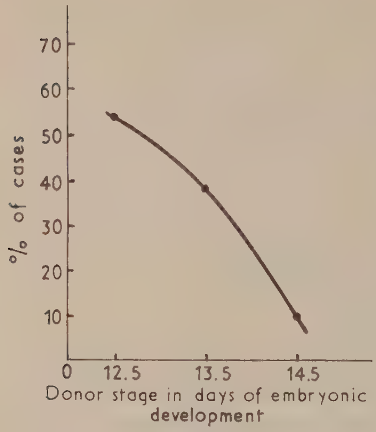

FIG. 1

Thus, this experimental series convincingly shows that (1) the external layer of the rat embryo eye rudiment is able to undergo transformation into retina over its whole area, though most often this transformation occurs at either the pupillar margin or that of the choroid fissure; (2) such transformation may proceed in the presence of the surrounding mesenchyme; and (3) when cultivating eye rudiments with the surrounding tissues, the capacity of the external layer to undergo transformation into retina decreases with age (Text-fig. 1); proliferation of the cells of the external layer sometimes leads to the doubling of the pigmented epithelium in implants of the two older stages.

D. *External layer transformation into retina in entire embryos*

Among 20 microphthalmic embryos, 5 cases were found in which eyes displayed different degrees of transformation of the external layer into retina. Without going into details of these cases, the most interesting features should be noted. The retina may appear both on the dorsal (Plate 4, fig. D) and on the ventral side (Plate 4, figs. C, E), either on the pupillary margin or on that of the choroid fissure, or in the form of a small local area far from the marginal zones. Mitoses in such retinae, as well as in implants, are found on the surface which faces the primary eye cavity. No mesenchyme sheaths are laid down along the retina in the external layer. Differentiation of the internal layer lags behind when compared with the norm (Plate 4, fig. A). The vitreous cavity in all of them is either reduced or lacking, or unclosed owing to the open choroid fissure. No such deviations in the differentiation of the external layer were found in any other microphthalmic eyes possessing well-manifested and closed cavities (Plate 4, fig. B).

DISCUSSION

Just as is the case in amphibians (Lopashov, 1945, 1960) and Acipenserid fishes (Dabaghian, 1958, 1959), both the external and the internal layers give rise to retina if the rudiment cells in mammals are caused to aggregate. Eye rudiments always form aggregations of this kind in the absence of the mesenchyme in a liquid medium, the individuality of the layers being lost as they merge into a common mass. Unlike Anurans and Acipenserids, in which all layers, including even the outgrowths of visual cells sticking out of the external surface of the implant, continue to differentiate in such a retina upon cultivation in cavity fluids, no further differentiation occurs in mammals in this kind of aggregation; more than that, its growth is also arrested, and soon necrosis appears at its centre. At the same time, eye rudiments taken with the lens and the surrounding mesenchyme form normally segregated eye-cups, in the retinae of which no degenerative changes are observed during equivalent periods of cultivation. When only the internal layer and the lens are cultivated, there also differentiates a retina divided into layers and oriented without rosettes, more or less in the form of a cup around the lens. This rules out the possibility that the anterior chamber liquid has an injurious effect on the retina. Thus, if an aggregation of eye-rudiment cells is a prerequisite for the primary formation of the retina in mammals as well as in other vertebrates, its growth and further differentiation are not achieved out of the complex of formative movements of the eye rudiment. One of the most important factors in this process is the tension of the eye tissues. In the course of normal development this tension is mediated in at least 3 stages by forces acting (1) as the eye-cup forms in the presence of the lens, (2) as the choroid fissure closes, and (3) as the intra-ocular pressure rises after the closure of the choroid fissure. This tension differs in its action on the internal and external layers, owing to the fact that, being the surface layer of the rudiment, the latter is exposed to a greater tension than the internal one.

Let us summarize the data concerning the factors determining the differentiation of the pigmented epithelium.

1. The external layer of the eye, in the absence of the mesenchyme at the stage of the optic vesicle and eye-cup prior to its pigmentation, develops into retina; in most cases only those regions of it differentiate into pigmented epithelium which maintain their connexion with mesenchyme cells or host iris.
2. However, in the experiment in which eye rudiments were cultured with their surrounding mesenchyme, individual areas of the external layer developed into retina.
3. In the normal development of the line of rats used this kind of deviation in the development of the eye is never to be found.

The data from the first series, as well as half the cases of the second series, in which either normal pigmented epithelium differentiated during the organotypical development of the eye or the retina appeared in the external layer in the absence of the mesenchyme, justify the conclusion that the differentiation of the pigmented epithelium undoubtedly depends on its contact with mesenchymal tissue. At the same time, the facts noted in item 2 of the above summary throw doubt upon the absolute validity of such a correlation. Therefore, during the cultivation in the anterior chamber of an adult eye some conditions necessary for the surrounding mesenchyme to maintain normal differentiation of the external layer to its full extent are lacking.

What are these conditions? First of all our attention should be drawn to the fact that no normal blood circulation is established in the implants. It is probable that owing to this fact (Kemp, 1953; Kemp & Quinn, 1954) the vitreous cavity is never formed in them, and that therefore the intra-ocular pressure, the important morphogenetic role of which was experimentally proved by Coulombre (1956), is lacking. Two hypothetical schemes may be put forward for the interpretation of the phenomenon observed.

1. If intra-ocular turgor and mechanical tension of the eye-cup walls are lacking, the normal formation of mesenchyme coats around it does not proceed, and insufficiently close contact between the mesenchyme cells and the external layer of the eye-cup is the result. When such defects of the mesenchyme coat occur, areas of the external layer differentiate not into pigmented epithelium but into retina. This occurs above all in regions predisposed to this differentiation, namely, at the pupillary margin or on the margin of the choroid fissure. If this is true, then the formation of mesenchyme coats of the eye in the course of normal development is determined by the intra-ocular pressure, while the coats, in their turn, determine the differentiation of the pigmented epithelium as such.

2. The normal differentiation of the pigmented epithelium requires as a condition of its contact with the mesenchyme the mechanical tension of the external layer of the eye. When the intra-ocular pressure is lacking, the cells of the presumptive pigmented epithelium are not expanded into a single layer but remain in local aggregations, and then either the effect of the mesenchyme reaches only to the surface cells, which in this case differentiate into pigmented epithelium (Plate 2, fig. E), or else it cannot override the tendency of eye rudiment cells to differentiate into retina upon their aggregation (Lopashov, 1960).

Do the data obtained suggest an exhaustive answer to the questions raised? When taken together with the lens and the mesenchyme, eye rudiments successfully pass the first stage of cup formation in the conditions provided by the anterior chamber. The external layer of these eye rudiments, however, differs up to the onset of pigmentation (12·5 days, normally) from the internal one only in the number of rows of cells in each layer (9–10 in the internal and 2–4 in the external layer). The cells of both layers possess spindle-shaped nuclei with several nucleoli and are oriented perpendicularly to the surface of the layers; many mitoses are found to occur on the surface of the primary cavity in both layers. If individual areas of the external layer preserve the same structure which they possess at this stage, this would justify the conclusion that retinal areas are present in the pigmented epithelium. On the one hand, such thickness represents the aggregation minimum required for the formation of retina; on the other hand, the external layer is as yet not strongly bound to the mesenchyme, owing to which the latter rather easily separates from the former when 11·5 and 12·5-day-old eye rudiments are implanted. Transition to the subsequent stage is associated with the arrangement of the cells of the external layer into one row, and with the transformation of the former into cuboidal epithelium with spherical nuclei; this is achieved under the operation of tangential forces arising in the process of the closure of the choroid fissure simultaneously with the increase of the volume of the internal layer and the lens. This second stage in the implants is not always completely achieved, owing perhaps to growth retardation of both the internal layer and the lens. Because of this, large areas of the external layer sometimes differentiate into retina. The third stage, the establishment of the intra-ocular pressure, does not occur at all in implants to the anterior chamber. If an aggregation of external layer cells remains preserved somewhere (on the pupillary margin and on the margin of the choroid coat such aggregations remain up to the stage of 13·5 days) then it is retina which develops in these areas because of the weakening of the forces stretching the external layer. And lastly, in the implants taken at the stage of 14·5 days and in which, by this time, the entire external layer is stretched, its differentiation hardly suffers. The above-formulated concepts of the role of intra-ocular pressure in the regular make-up of the eye find morphological confirmation in the pictures of corresponding malformations of eyes in rat embryos obtained as a result of various actions of pregnant females (present communication; Giroud *et al.*, 1954; Giroud, 1957). Retardation of the closure of the choroid fissure is always accompanied by the appearance of retina in the external layer of the eye.

In connexion with the above remarks it is unnecessary to regard the high frequency of retina formation in marginal zones as due to their particular properties. The peculiarity of these zones consists only in the fact that the arrangement of their cells in one layer takes place later than in other regions of the external layer. Since retina appears on both dorsal and ventral sides to an equal degree, this rules out the assumption that in the external layer of the eye rudiment of mammals a gradient of morphogenetic potentials exists analogous to that shown for lens formation in Urodelan larvae (Sato, 1951) and for the retina in Anuran larvae (Lopashov, 1955; Stroeva, 1956*a*).

As to the mesenchyme, its determining role for the pigmented epithelium as a whole operates in the presence of forces stretching the external layer; with its transformation into the monolayered epithelium the relative role of mesenchyme increases with the simultaneous increase of contact between the mesenchyme and the external layer, assisted by the increasing stickiness of the latter. The impression is gained that the mesenchyme stabilizes the epithelial state of the latter and inhibits mitoses (analogous to its action on the tissue of the neural plate, Takaya, 1956); the action of the mesenchyme being eliminated, the external layer proliferates and gives rise to retina, at least at the stage of 13·5 days. It is possible that the age-dependent decrease of the ability of the external layer to differentiate into retina at the stage of 14·5 days is simply determined by the closeness of its contact with the mesenchyme coats, and not yet by the inhibition of its own morphogenetic potentialities.

Does the presence of the mesenchyme surrounding the eye rudiment and participating in the determination of the pigmented epithelium differentiation guarantee choroid coat formation? Apparently not. Both in the case of hereditary (Mann 1957) and experimentally induced (present communication; Giroud *et al.*, 1954; Giroud, 1957) colobomas either the entire choroid coat or its chorio-capillary layer and Bruch membrane are lacking in front of the retinal areas in the external layer, in spite of the surrounding mesenchyme. Is Mann (1957) right in claiming that the formation of the mesenchyme coats of the eye is determined by the normal pigmented epithelium? Does the stickiness of the external layer, which appears with the onset of pigmentation, play some role in it? Most likely both questions must be answered affirmatively, though more experimental evidence is necessary. Independently of the solution of this problem one can assume that the intra-ocular pressure plays a role in the determination of the normal development of the pigmented epithelium in mammals, as well as in the determination of eye size, the formation of the ciliary body, and the determination of the size and curvature of the cornea in birds (Coulombre, 1956, 1957; Coulombre & Coulombre, 1957, 1958).

TABLE 3

Changes of morphogenetic potentialities of chick eye-rudiments

Somite number	*Incubation time*	*Stage of eye formation*	*Morphogenetic potentialities of the external and internal layers of the eye*
5	—	Appearance of the optic vesicle (Alexander, 1937)	—
9	33 hours	Optic vesicle comes into contact with the ectoderm (Alexander, 1937)	—
20	—	—	After this stage the external layer cannot restore the entire eye (Reinbold, 1958)
21	—	Optic vesicle becomes a cup with open choroid fissure (Reinbold, 1958) (Alexander, 1937)	Formation of typical and atypical colobomas of the retina (Gayer, 1942)
23	—		
24	48 hours	—	—
35	72 hours	—	Up to this stage the ability of the external layer to develop the retina is preserved (Alexander, 1937)
36			
—	76–80 hours	—	The ability of the retina to develop the pigmented epithelium is lost (Dorris, 1938)
45	The middle of the 4th day	Pigmentation of the external layer begins (Alexander, 1937) Mitotic activity in the pigmented epithelium ceases (Coulombre, 1955)	—
	5th day	Closure of the choroid fissure (Gayer, 1942)	—

In birds, determination of the pigmented epithelium is shifted to earlier stages of eye development. As can be seen from Table 3, the ability of the external layer to transform into retina is lost considerably earlier than the initiation of its pigmentation and prior to the closure of the choroid fissure. The intra-ocular pressure in birds cannot, therefore, play such an important role in the differentiation of the pigmented epithelium as it does in mammals. Owing to just this fact only Gayer (1942), when working on early stages, succeeded in observing retina formation in the external layer and in discovering a correlation between this phenomenon and the extent of development of mesenchymal eye-coats. In other experiments, in which the eye rudiments used were taken at later developmental stages (Reinbold, 1954; Coulombre, 1956), no deviations in the pigmented epithelium differentiation could be observed.

The experiments on mammals complete an investigation cycle carried out in order to elucidate the conditions under which neural parts of the eye rudiment are formed in the representatives of the main systematic groups of vertebrates. This allows us to compare certain features in the mechanism of eye formation in a series of forms. It should be noted that, although the process is made up of similar components in all groups, the interaction of these components is achieved in somewhat different ways in each individual group, and the specific importance of each component alters, even though a similar final result is achieved. This should be taken into consideration when analysing morphogenetic relations in new species. Regularities found in some forms cannot be automatically applied to others, but they may serve as a key to new analyses.

SUMMARY

1. The conditions of retina and of pigmented epithelium formation in mammals were studied in experiments on the cultivation of the rat eye rudiments at the stage of eye vesicle (11·5 days) and that of the eye-cup prior to the onset of pigmentation of its external layer (12·5 days), at the onset of its pigmentation, and at the closure of the choroid fissure (13·5 and 14·5 days), in the anterior chamber of the adult eye.

2. In the absence of mesenchyme, eye rudiments taken at the stage of 11·5 and 12·5 days develop completely into the retina; they become arranged in rosettes, their growth slows down, and no further differentiation takes place. Pigmented epithelium areas arise only when the eye rudiment is in contact with either the mesenchyme or the host iris.

3. When cultivating the internal layer with the lens, the retina becomes arranged in the form of a cup surrounding the lens; no rosettes are formed, and the retina differentiates into layers.

4. Eye rudiments at all four stages, when cultivated together with the lens and the mesenchyme, develop organotypically, but no vitreous cavity is formed in them. In one half of the implants of this series normal pigmented epithelium was differentiated, while in another half individual areas of the external layer

were transformed into retina. The ability of the external layer of the eye to undergo transformation into retina decreases with age.

5. This type of deviation in the external layer development is never to be found in the normal development of the line of rats used.

6. Histological analysis of microphthalmic eyes obtained as the result of the action of X-rays or of thermal shock on pregnant females showed that the absence of the closure of the vitreous cavity was accompanied by the development of retina in the external layer.

7. Our data allow us to draw the following conclusions. The external layer of the mammalian eye is capable of differentiating into retina up to the stage of the onset of pigmentation. The determining role of the mesenchyme in the differentiation of the external layer is realized in the presence of forces stretching this layer. These forces arise during the closure of the choroid fissure and upon the appearance of the intra-ocular pressure after its closure. Not only is the organotypical development of the eye (cup formation, segregation into the retina and the pigmented epithelium) determined by the interaction of the neural anlage of the eye with the lens and the mesenchyme, but also the growth and differentiation of the retina are directly correlated with the entire complex of formative movements characterizing the normal development of the eye.

ВЫВОДЫ

1. В опытах культивации зачатков глаз крыс на стадиях глазного пузыря (11, 5 суток), глазного бокала до начала пигментации его наружного листка (12, 5 суток), начала его пигментации и закрытия глазной щели (13, 5 и 14, 5 суток) в передней камере взрослого глаза исследовались условия формирования сетчатки и пигментного эпителия у млекопитающих.

2. В отсутствии мезенхимы зачатки глаз, взятые на стадиях 11, 5 и 12, 5 суток, развиваются целиком в сетчатку; она располагается розетками, её рост задерживается, и дифференцировка не наступает. Участки пигментного эпителия возникают лишь при контакте зачатка глаза с мезенхимой или радужиной хозяина.

3. При культивации внутреннего листка с линзой сетчатка располагается бокалообразно вокруг последней, не образуется розеток, и она дифференцируется на слои.

4. Зачатки глаз всех четырех стадий, культивируемые вместе с линзой и мезенхимой, развиваются органотипически, но у них никогда не образуется полость стекловидного тела. В одной половине случаев в этой серии дифференцировался нормальный пигментный эпителий, в другой — отдельные участки наружного листка превратились в сетчатку. Способность наружного листка глаза к превращению в сетчатку с возрастом падает.

5. Никогда подобные уклоненения в развитии наружного листка не встречаются в нормальном развитии данной линии крыс.

6. Гистологический анализ микрофталмичных глаз, полученных в результате действия X-лучей или теплового шока на беременную самку, показал, что случаи отсутствия или незамкнутости полости стекловидного тела сопровождаются возникновением сетчатки в составе наружного листка.

7. Наши данные позволяют заключить следующее: наружный листок глаза млекопитающих способен дифференцироваться в сетчатку вплоть до стадии начала пигментации. Детерминирующая роль мезенхимы в его дифференцировке осуществляется при наличии сил, растягивающих наружный листок в однослойный эпителий. Эти силы возникают в процессе закрытия глазной щели и при появлении внутриглазного давления после её закрытия. Не только органотипическая организация глаза (бокалообразование, расчленение на сетчатку и пигментный эпителий) определяется взаимодействием его нейральной закладки с линзой и мезенхимой, но и рост и дифференцировка сетчатки также находится в прямой зависимости от всего комплекса формообразовательных движений, характеризующих нормальное развитие глаза.

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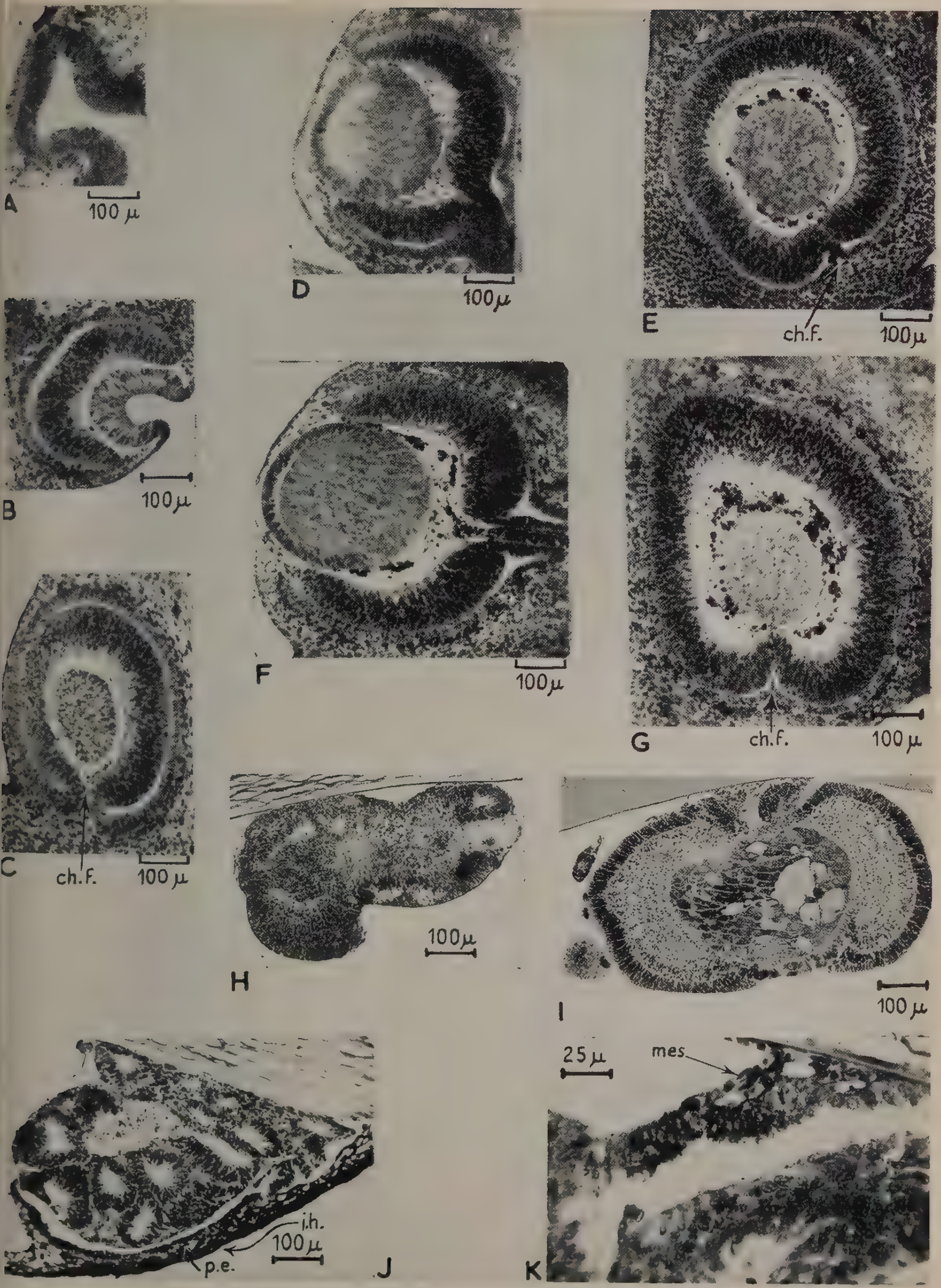

O. G. STROEVA

Plate 1

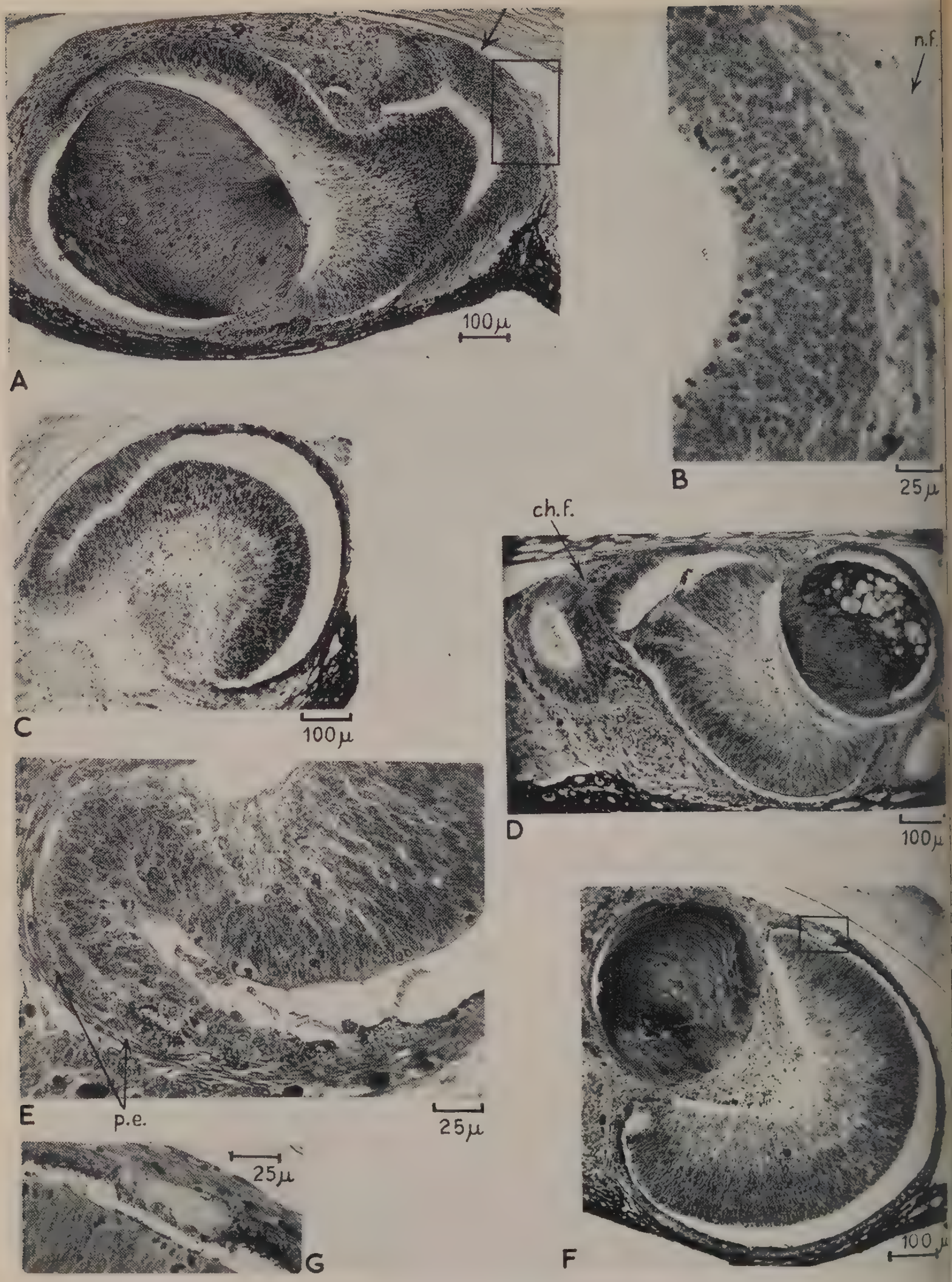

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Plate 2

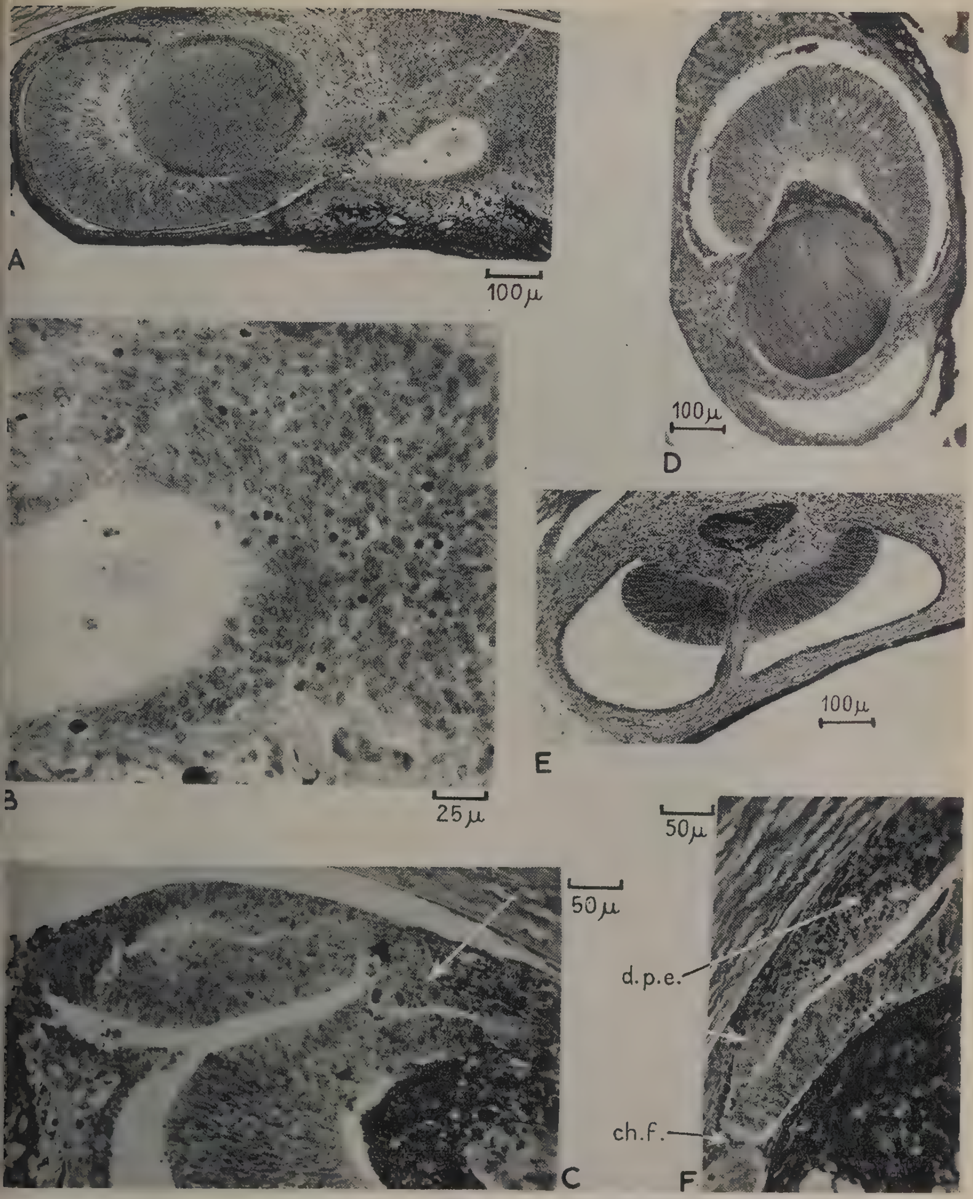

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Plate 3

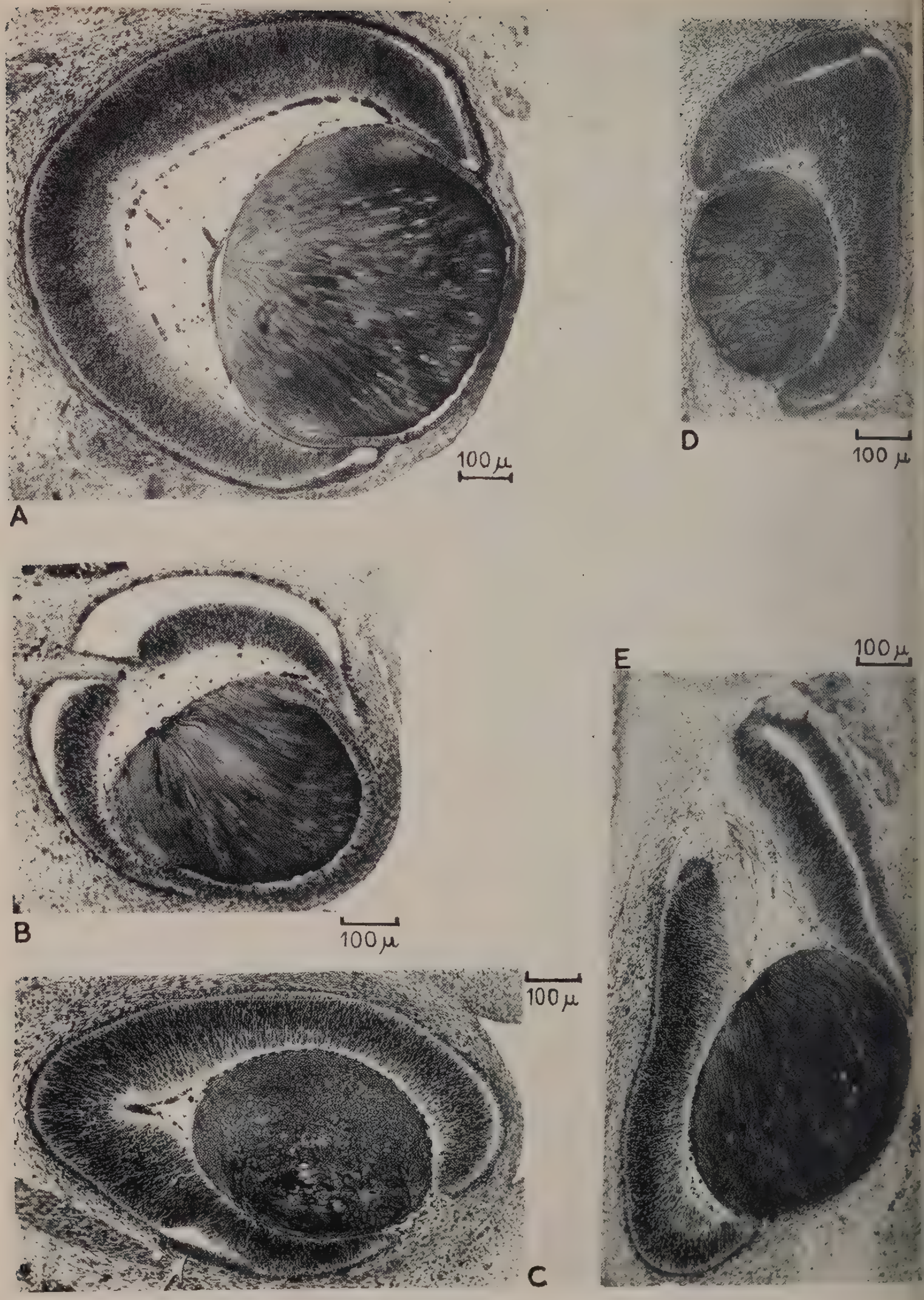

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Plate 4

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EXPLANATION OF PLATES

PLATE 1

FIGS. A–G. Stages in the normal development of donors.

FIG. A. Optic vesicle (11·5 days).

FIGS. B & C. Eye-cup prior to the onset of the external layer pigmentation (12·5 days); *ch.f.* = choroid fissure.

FIGS. D & E. Eye-cup after the onset of the external layer pigmentation (13·5 days).

FIGS. F & G. Eye-cup with the closed choroid fissure (14·5 days).

FIG. H. Eye rudiment taken at the stage of 12·5 days without mesenchyme; the entire eye rudiment consists of the retinal tissue (8 days of cultivation).

FIG. I. Internal layer together with the lens, taken at the stage of 12·5 days. Internal retina layer has loosened (31 days of cultivation).

FIG. J. Eye rudiment taken at the stage of 12·5 days without mesenchyme; at the side of contact with the host iris (*i.h.*) the pigmented epithelium (*p.e.*) has differentiated; necrosis is to be seen in the centre of the retina (7 days of cultivation).

FIG. K. Pigmented epithelium section which has arisen at the contact with the mesenchyme cells (*mes.*) in the implant taken at the stage of 12·5 days, after the removal of the mesenchyme (6 days of cultivation).

PLATE 2

FIG. A. Eye rudiment taken at the stage of 12·5 days with the surrounding tissues; the mesenchyme escaped at the posterior pole, and here the external layer differentiated into the retina (shown by arrow; 8 days of cultivation).

FIG. B. Detail of the external layer taken from the square in fig. A; *n.f.* = nerve fibres.

FIG. C. Retina in the external layer in the presence of mesenchyme on the pupillary margin of the implant taken at the stage of 12·5 days with the surrounding tissues (7 days of cultivation).

FIG. D. Retina on the margin of the choroid fissure (*ch.f.*), in the presence of mesenchyme in the implant taken at the stage of 12·5 days with the surrounding tissues (7 days of cultivation).

FIG. E. Formation of pigmented epithelium areas (shown by arrow) on the external surface of the retina; these arose in the external eye layer on the pupillar margin in the implant taken at the stage of 12·5 days, with the surrounding tissues (8 days of cultivation).

FIG. F. Implant taken at the stage of 12·5 days with the surrounding tissues, normally differentiated into retina and pigmented epithelium (7 days of cultivation).

FIG. G. Detail of the external layer section taken from the square in fig. F, which differentiated into a nerve fragment.

PLATE 3

FIG. A. Implant taken at the stage of 11·5 days with the surrounding tissues; the arrow shows the area of the external layer, which acquired a neural character, and from which the neural mesenchyme arose (6 days of cultivation).

FIG. B. The same section as in fig. A under high magnification.

FIG. C. Implant taken at the stage of 13·5 days with the surrounding tissues; between the arrows marking the normal pigmented epithelium lies an area on the retina which arose here after the mesenchyme lifted off (4 days of cultivation).

FIG. D. Implant taken at the stage of 13·5 days with the surrounding tissues, normally differentiated into retina and pigmented epithelium (4 days of cultivation).

FIG. E. Implant taken at the stage of 14·5 days with the surrounding tissues, normally differentiated into retina and pigmented epithelium. Retina growth is strongly retarded (4 days of cultivation).

FIG. F. Implant taken at the stage of 14·5 days with the surrounding tissues. The arrow marks the retinal formation on the margin of the choroid fissure (*ch.f.*); *d.p.e.* = doubled pigmented epithelium (4 days of cultivation).

PLATE 4

FIGS. A–C. Normal and altered eyes of 17-day-old whole rat embryos.

FIG. A. Normal eye.

FIG. B. Microphthalmic eye normally differentiated into retina and pigmented epithelium.

FIG. C. Retina (shown by the arrow) developed in the external layer on the ventral side of the eye. Eye cavity reduced.

FIG. D. Retina developed in the external layer of the eye on the dorsal side. Eye cavity absent.

FIG. E. Retina developed on the ventral side of the eye. Eye cavity unclosed owing to the open choroid fissure.

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